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SEAWEEDS FROM THE PORTUGUESE COAST: CHEMISTRY, ANTIMICROBIAL AND ANTI-INFLAMMATORY CAPACITY

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Phytochemistry and Pharmacognosy Speciality

Work performed under the supervision of

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and co-supervision of

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“When we least expect it, we find what we have looked for during our entire life.”

To my parents and brother

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2. Lopes G, Sousa C, Silva L.R, Pinto E, Andrade PB, Valentão P. Can phlorotannins extracts constitute a novel pharmacological alternative for microbial infections with associated inflammatory conditions? PLOS ONE 2012 Feb; 7 (2): e31145.

3. Ferreres F, **Lopes G**, Izquierdo AG, Andrade PB, Sousa C, Mouga T, Valentão P. Phlorotannins extracts from Fucales characterized by HPLC-DAD-ESI-MSn: Approaches to hyaluronidase inhibitory capacity and antioxidant properties. Mar Drugs 2012 Dec; 10 (12): 2766-2781.

4. Lopes G, Pinto E, Andrade PB, Valentão P. Antifungal activity of phlorotannins against dermatophytes and yeasts: Approaches to the mechanism of action and influence on *Candida albicans* virulence factor. PLOS ONE 2013 Aug; 8(8): e72203.

5. Lopes G, Daletos G, Proksch P, Andrade PB, Valentão P. Anti-inflammatory potential of monogalactosyl diacylglycerols and a glycerolipid from the edible brown algae *Fucus spiralis* Linnaeus. (*Submitted*).

Book chapters:

Lopes G, Sousa C, Valentão P, Andrade PB (2012). Sterols in algae and health. In: Hernandez-Ledesma B, Herrero M (Eds.), Bioactive compounds from marine foods: plant and animal sources. Wiley-Blackwell, Oxford, UK. 320 pp. ISBN: 978-1-118-41284-8

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Oral communications:

1. Lopes G, Sousa C, Bernardo J, Mouga T, Andrade PB, Valentão P. Phytosterols profile of edible macro algae. **IJUP – Encontro de Jovens Investigadores da Universidade do Porto.** 17th to 19th February 2010, Reitoria da Universidade do Porto.

2. Lopes G, Sousa,C, Bernardo J, Mouga T, Andrade PB, Valentão P. Fitosteróis na autenticidade de macroalgas comestíveis. **Autenticidade de Produtos Alimentares.** 12th and 13th March 2010, Instituto Politécnico de Bragança.

3. Lopes G, Silva LR, Sousa C, Pinto E, Mouga T, Andrade PB, Valentão P. Anti-inflammatory and antimicrobial activities of phlorotannins purified extracts from brown seaweeds collected in the Portuguese coast. **2ª Workshop Anual Bioplant.** 18th April 2011, Universidade do Minho.

4. Lopes G, Pinto E, Andrade PB, Mouga T, Valentão P. Antifungal activity and mechanism of action of phlorotannins in *Candida albicans* yeast: possible role in the respiratory chain and induced oxidative stress. **3ª Workshop Anual Bioplant.** 4th to 6th December 2012, Universidade de Aveiro.

Poster communications:

1. Lopes G, Sousa C, Bernardo J, Mouga T, Andrade PB, Valentão P. Phorotannins in edible macro algae purified extracts. **1º Workshop Anual Bioplant.** 29th and 30th March 2010, Faculdade de Ciências da Universidade do Porto.

2. Lopes G, Leitão-Rocha A, Santos A, Almeida C, Costa D, Costa DP, Magalhães M, Barros SV. *Cystoseira tamariscifolia* (Hudson) Papenfuss (Phaeophyta): an alternative to plants? **IJUP – Encontro de Jovens Investigadores da Universidade do Porto.** 22th to 24th February 2012, Reitoria da Universidade do Porto.

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4. Mendo T, **Lopes G**, Andrade PB, Valentão P. Metabolites from brown seaweeds with promising biological activities. **IJUP – Encontro de Jovens Investigadores da Universidade do Porto.** 13th to 15th February 2013, Reitoria da Universidade do Porto.

5. Lopes G, Freitas A, Rocha L, Machado N, Ferreira R, Pereira R, Silva ST, Martins T, Guimarães V. Marines do it better: the case of *Cystoseira usneoides* (Linnaeus) M. Roberts (Phaeophyta). **IJUP – Encontro de Jovens Investigadores da Universidade do Porto.** 13th to 15th February 2013, Reitoria da Universidade do Porto.

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7. Lopes G, Daletos G, Procksh P, Andrade PB, Valentão P. Monogalactosyl diacylglycerols from the edible brown algae *Fucus spiralis* Linnaeus: Anti-inflammatory potential on RAW 264.7 macrophage cell line. **4ª Workshop Anual Bioplant.** 18th and 19th July 2013, Faculdade de Ciências da Universidade do Porto.

AUTHOR'S DECLARATION

The author declares that she has actively participated in the collection and study of the material included on all works and written all the manuscripts included in this dissertation, with the collaboration of other coauthors.

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RESUMO

RESUMO

Os oceanos compreendem uma imensa diversidade de organismos vivos, ainda pouco explorados quando comparados com os organismos terrestres. As algas são parte dessa diversidade, constituindo um dos mais importantes grupos de organismos, tanto em número como em variedade de espécies. Atendendo à pesquisa constante de compostos bioativos, as algas atraíram a atenção dos cientistas e tornaram-se objeto de muitos estudos nas últimas décadas. Portugal tem uma localização geográfica privilegiada, com uma vasta área costeira e uma gama de temperatura e exposição solar propícias ao desenvolvimento de várias espécies de algas. Com o objetivo de valorizar este recurso natural, dezoito espécies de algas colhidas na costa oeste Portuguesa pertencentes a três filos diferentes (Chlorophyta, Rhodophyta e Phaeophyta), foram exploradas no que respeita à sua composição química e atividade biológica.

O perfil de esteróis das amostras foi analisado por HPLC-DAD, permitindo a identificação e quantificação de 7 compostos: desmosterol, ergosterol, fucosterol, colesterol, campesterol, estigmasterol e β-sitosterol. As espécies pertencentes aos filos Chlorophyta e Phaeophyta caracterizaram-se pela presença maioritária de esteróis C_{29}, sendo o isofucosterol/fucosterol os principais compostos, respectivamente, enquanto as espécies do filo Rhodophyta se caracterizaram pela sua abundância em colesterol. As espécies dos filos Chlorophyta e Phaeophyta apresentaram o maior teor de esteróis, sendo a alga castanha *Cystoseira tamariscifolia* Hudson Papenfuss a espécie mais rica. Considerando os efeitos benéficos dos esteróis para a saúde humana, e tendo em conta o perfil qualitativo e quantitativo apresentado pelas espécies em estudo, as pertencentes aos filos Chlorophyta e Phaeophyta revelaram-se mais promissoras.

As espécies de algas de Phaeophyta foram analisadas relativamente ao seu teor total em florotaninos, determinado num extrato purificado pelo método específico do dimetoxibenzaldeído. A espécie *Fucus spiralis* Linnaeus apresentou a maior quantidade destes compostos, seguida pelas espécies do género *Cystoseira*. Foram avaliadas as atividades anti-inflamatória, antimicrobiana e a capacidade de sequestro do óxido nítrico (NO) dos extratos purificados de florotaninos. A ação anti-inflamatória foi estudada através da avaliação da capacidade dos extratos para reduzir o NO produzido pela linha celular de macrófagos RAW 264.7. A espécie *C. tamariscifolia* apresentou a melhor atividade, sem toxicidade nas concentrações testadas. A atividade sequestrante para o NO foi avaliada também num sistema não celular, tendo os melhores resultados sido obtidos com o extrato de *F. spiralis*. A atividade antimicrobiana dos extratos foi avaliada relativamente a uma vasta gama de bactérias e fungos patogénicos, sendo mais ativos

contra bactérias Gram$^+$ e dermatófitos, com destaque para as espécies *F. spiralis* e *Cystoseira nodicaulis* (Withering) M. Roberts.

O perfil de florotaninos das algas mais promissoras, ou seja, aquelas com maiores quantidades de florotaninos e melhores atividades biológicas, foi analisado por HPLC-DAD-ESI/MS^n. Foram caracterizados 22 florotaninos nas espécies estudadas, pertencentes às classes ecol e fucofloroetol: 8 em *C. nodicaulis*, 2 em *C. tamariscifolia*, 4 em *Cystoseira usneoides* (Linnaeus) M. Roberts e 8 em *F. spiralis*. A capacidade dos extratos de florotaninos destas espécies para sequestrar o radical anião superóxido, para inibir a peroxidação lipídica e para prevenir a degradação do ácido hialurónico, por meio da inibição da hialuronidase, foi determinada. Todas as espécies estudadas apresentaram resultados promissores, sendo a *F. spiralis* a mais ativa.

Tendo em conta o aparecimento crescente de resistências a antifúngicos, a atividade antifúngica de extratos purificados de florotaninos foi aprofundada, relativamente a uma variedade mais alargada de leveduras e dermatófitos, demonstrando atividade fungistática contra leveduras e fungicida contra dermatófitos. O mecanismo de ação dos extratos purificados de florotaninos foi também avaliado, usando a levedura *Candida albicans* e o dermatófito *Trichophytom rubrum* como modelos, e baseou-se no seu efeito sobre os alvos mais comuns dos fungos. Embora os florotaninos não apresentem um efeito claro sobre os componentes da membrana e parede celular dos fungos, o seu efeito sobre a rede metabólica dos microorganismos é notório. Adicionalmente, a espécie *F. spiralis* apresentou resultados promissores na inibição do fator de virulência de *C. albicans*.

Dado o potencial químico e biológico da espécie *F. spiralis*, o seu estudo foi aprofundado e prosseguiu-se com o isolamento de compostos. Dois monogalactosil diacilgliceróis (MGDGs) e um monoacilglicerol foram isolados por métodos cromatográficos e as suas estruturas foram elucidadas por meio de espectroscopia (RMN e MS) e por comparação com a literatura. O monoacilglicerol, composto por glicerol ligado a ácido oleico (C18:1 Ω9) e os MGDGs, compostos por uma porção de glicerol ligado a uma unidade de galactose e de ácido eicosapentaenoico (C20:5 Ω3) combinado com o ácido linolénico (C18:3 Ω3) ou ácido octadecatetraenoico (C18:4 Ω3), respetivamente, foram testados quanto à sua atividade citotóxica e anti -inflamatória em macrófagos RAW 264.7, apresentando capacidade para inibir a produção de NO em concentrações não citotóxicas.

As algas estudadas apresentaram características biológicas interessantes, sendo as espécies do género *Cystoseira* e o *F. spiralis* particularmente promissoras para o possível desenvolvimento de formulações farmacêuticas e de alimentos funcionais.

Palavras-chave: Atividade anti-inflamatória; atividade antimicrobiana; atividade antioxidante; esteróis; florotaninos; glicerolípidos; macroalgas.

ABSTRACT

ABSTRACT

Oceans comprise an immense diversity of living organisms, still underexplored if compared with terrestrial ones. Seaweeds are part of this diversity, constituting one of the most important groups of organisms, in both number and variety of species. Considering the constant search for new bioactive compounds, seaweeds have attracted scientists' attention and became the subject of many studies in the last decades. Portugal has a privileged geographical location, with a wide coastal area and a range of temperature and sun exposition propitious to the development of various species of seaweeds. With the aim of valuing this natural resource, seaweeds species belonging to three different phyla (Chlorophyta, Rhodophyta and Phaeophyta) were analyzed and explored for their chemical composition and biological activities.

The sterols profile of eighteen seaweeds was analyzed by HPLC-DAD, allowing the identification and quantification of 7 compounds: desmosterol, ergosterol, fucosterol, cholesterol, campesterol, stigmasterol and β-sitosterol. Species belonging to Chlorophyta and Phaeophyta contained mainly C_{29} sterols, isofucosterol/fucosterol being the major compounds, respectively, while the ones of Rhodophyta where characterized by their abundance in cholesterol. Chlorophyta and Phaeophyta presented the highest total sterols content, the brown seaweed *Cystoseira tamariscifolia* Hudson Papenfuss being the richest species. Considering the sterols health effects and the qualitative and quantitative profile presented by the studied seaweeds, Chlorophyta and Phaeophyta represent the most promising groups.

Phaeophyta species were analyzed for their total phlorotannins content by their quantification in a purified phlorotannins extract using the dimethoxybenzaldehyde specific assay. Of the studied species, *Fucus spiralis* Linnaeus presented the highest total phlorotannins amount, followed by the *Cystoseira* species. The anti-inflammatory, antimicrobial and NO scavenging activity of the purified extracts was analyzed. The anti-inflammatory capacity was studied by evaluating the capacity of the extracts to reduce the NO produced by RAW 264.7 macrophage cells, *C. tamariscifolia* presenting the best activity, with no citotoxicity under the tested concentrations. The NO scavenging activity was evaluated on a cell-free system, *F. spiralis* presenting the best results. The antimicrobial activity was evaluated over a wide range of pathogenic $Gram^+$ and $Gram^-$ bacteria and fungi. In a general way, phlorotannins extracts were more active against $Gram^+$ bacteria and, among fungi, against dermatophytes, with emphasis on *F. spiralis* and *Cystoseira nodicaulis* (Withering) M. Roberts.

The phlorotannins profile of the most promising seaweeds, i.e., the ones with higher phlorotannins amounts and better biological activities, was analyzed by HPLC-DAD-ESI/MS^n. Twenty two different phlorotannins belonging to eckol and fucophlorethol main groups were characterized in the studied seaweeds: 8 in *C. nodicaulis*, 2 in *C. tamariscifolia*, 4 in *Cystoseira usneoides* (Linnaeus) M. Roberts and 8 in *F. spiralis*. The capacity of the phlorotannins extracts of these species to sequester superoxide anion radical, to inhibit lipid peroxidation and to prevent the degradation of hyaluronic acid, by the inhibition of hyaluronidase, was determined. All of the studied species presented promising results; nevertheless, *F. spiralis* was, by far, the most active species.

Taking into account the emergence of resistance to antifungal drugs, the antifungal activity of purified phlorotannins extracts was extended to a larger number of yeasts and dermatophytes, demonstrating fungistatic activity against yeast and fungicidal capacity against dermatophytes. The mechanism of action of purified phlorotannins extracts was also evaluated, using the yeast *Candida albicans* and the dermatophyte *Trichophytom rubrum* as models. The effect of purified phlorotannins extracts was evaluated over the most common fungal targets. Although phlorotannins did not present a clear effect on fungal cell membrane and cell wall, they clearly interacted with the microorganism's metabolic network. Additionally, *F. spiralis* presented promising results on the inhibition of *C. albicans* virulence factor.

Considering the potential of the seaweed species *F. spiralis*, the studies were deepened and continued with the isolation of compounds. Two monogalactosyl diacylglicerols (MGDGs) and one monoacylglycerol were isolated by chromatographic methods and their structures were elucidated by spectroscopic means (NMR and MS) and by comparison with the literature. The monoacylglycerol was composed of a glycerol moiety linked to oleic acid (C18:1 Ω9) and the MGDGs contained a glycerol moiety linked to a galactose unit and eicosapentaenoic acid (C20:5 Ω3) combined with linolenic acid (C18:3 Ω3) or octadecatetraenoic acid (C18:4 Ω3), respectively. The isolated compounds were tested for their cytotoxic and anti-inflammatory activity in RAW 264.7 macrophage cells, presenting the capacity to inhibit NO production at non-cytotoxic concentrations.

The studied seaweeds demonstrated interesting nutritional and biological features, *Cystoseira* species and *F. spiralis* being particularly promising for the possible development of pharmaceutical formulations and functional food products.

Keywords: Anti-inflammatory activity; antimicrobial activity; antioxidant activity; glycerolipids; phlorotannins; sterols; seaweeds.

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ABBREVIATIONS, ACRONYMS AND SYMBOLS

ABBREVIATIONS, ACRONYMS AND SYMBOLS

Ac-CoA	Acetyl-coenzyme A
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photo-ionization
ARA	Arachidonic acid
ATCC	American type culture collection
ATP	Adenosine triphosphate
BHT	*Tert*-butyl-4-hydroxytoluene
BSA	Bovine serum albumin
BuOH	n-Butanol
Ca^{2+}	Calcium
CAT	Catalase
CC	Column chromatography
CDP-ME	4-Diphosphocytidyl-2-*C*-methyl-D-erythritol
CDP-MEP	4-Diphosphocytidyl-2-*C*-methyl-D-erythritol-2-phosphate
CHD	Cardiovascular heart disease
CLSI	Clinical and Laboratory Standards Institute
CMK	CDP-ME kinase
CNS	Central nervous system
CO_2	Carbon dioxide
CoA	Coenzyme A
DAD	Diode array detection
DGDGs	Digalactosyldiacylglycerols
DMAB	4-Dimethylaminobenzaldehyde
DMAPP	Dimethylallyl diphosphate
DMBA	2,4-Dimethoxybenzaldehyde
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOXP	1-Deoxy-D-xylulose-5-phosphate

DPBS	Dulbecco's phosphate buffered saline
DXR	DOXP reductoisomerase
DXS	DOXP synthase
em	Emission wavelength
EPA	Eicosapentaenoic acid
ESI	Electron spray ionization
ETOAc	Ethyl acetate
ex	Excitation wavelength
F	Fluorescence
FBS	Heat inactivated fetal bovine serum
Fe^{2+}	Cation iron II
$FeCl_3$	Ferric chloride
$FeSO_4.7H_2O$	Iron (II) sulfate hepta hydrated
FF	Faculdade de Farmácia
GAP	D-Glyceraldehyde-3-phosphate
GC	Gas chromatography
GPx	Glutathione peroxidase
$Gram^+$	Gram positive bacteria
$Gram^-$	Gram negative bacteria
GSH	Glutathione
H_2O	Water
H_2O_2	Hydrogen peroxide
H_3PO_4	*ortho*-Phosphoric acid
HA	Hyaluronic acid
HAase	Hyaluronidase
HCl	Hydrochloric acid
HDL	High density lipoproteins
HMBPP	1-Hydroxy-2-methyl-2-(*E*)-butenyl-4- diphosphate
HMG-CoA	3-(*S*)-Hydroxy-3-methylglutaryl-CoA
HOCl	Hypochlorous acid

HPLC	High-performance liquid chromatography
IDI	Isopentenyl-diphosphate isomerase
iNOS (NOS II)	Inducible nitric oxide synthase
IPP	Isopentenyl diphosphate
KHSO4	Potassium hydrogen sulfate
KOH	Potassium hydroxide
LA	Linoleic acid
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LDH	Lactate dehydrogenase
LDL	Low density lipoproteins
LNA	α-Linolenic acid
LOD	Limit of detection
LOQ	Limit of quantification
LPO	Lipid peroxidation
LPS	Lipopolysaccharide
m/z	Mass to charge
MALDY	Matrix-assisted laser desorption ionization
MCT	MEP cytidyl transferase
MEcPP	2-*C*-Methyl-D-erythritol 2,4-cyclodiphosphate
MeOH	Methanol
MEP	2-*C*-Methyl-D-erythritol-4-phosphate
MGDGs	Monogalactosyldiacylglycerols
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth
MIC	Minimum inhibitory concentration
MK	Mevalonate kinase
MLC	Minimum lethal concentration
MOPS	3-(N-Morpholino) propanesulfonic acid
MS	Mass spectrometry

MTT	Thiazolyl blue tetrazolium bromide
MVA	Mevalonate
MVADP	Mevalonate-5-diphosphate
NaCl	Sodium chloride
NADH	β-Nicotinamide adenine dinucleotide reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate reduced form
NaNO2	Sodium nitrite
NaOH	Sodium hydroxide
NBT	Nitrotetrazolium blue chloride
NH4	Ammonium
NIM	Negative ion mode
NMR	Nuclear magnetic resonance
$^{\cdot}NO$	Nitric oxide radical
NO	Nitric oxide
NO^{-}	Nitroxyl anion
NO^{+}	Nitrosonium cation
NO_2^{-}	Nitrite
NOS	Nitric oxide synthase
NP	Normal-phase
O_2	Oxygen
$O_2^{\cdot -}$	Superoxide anion radical
$^1O_2$	Singlet oxygen
$^3O_2$	Triplet molecular oxygen
OH	Hydroxyl
$^{\cdot}OH$	Hydroxyl radical
$ONOO^{-}$	Peroxynitrite
ONOOH	Peroxynitrous acid
PGE_2	Prostaglandin E2
PIM	Positive ion mode
PMK	Phosphomevalonate kinase

PMS	Phenazine methosulfate
PPMD	Mevalonate-5-diphosphate-decarboxylase
PUFAs	Polyunsaturated fatty acids
RAW 264.7	Murine macrophage-like cell line
RDS	Relative standard deviation
Rf	Retention factor
RHO	Rhodamine 123
RNS	Reactive nitrogen species
RO$^{\cdot}$	Alkoxyl radical
ROO$^{\cdot}$	Peroxyl radical
ROS	Reactive oxygen species
RP	Reversed-phase
RPMI	Roswell Park Memorial Institute 1640 Medium
SDA	Stearidonic acid
SDA	Sabouraud dextrose agar
SMT	Sterol methyltransferase
SNP	Sodium nitroprusside dehydrate
SOD	Superoxide dismutase
SPE	Solid-phase extraction
TLC	Thin-layer chromatography
UV	Ultraviolet
Vis	Visible
VLC	Vacuum liquid chromatography
XO	Xanthine oxidase

Units of Measure

Å	Ångström (1 Å = 10^{-10} m)
Abs	Absorbance
CFU	Colony forming units

ºC	Celsius degree
MFA	MacFarland
Da	Dalton
kDa	Kilodalton
µg	Microgram
mg	Milligram
g	Gram
Kg	Kilogram
µL	Microlitre
mL	Millilitre
µM	Micromolar
mM	Milimolar
M	Molar
nm	Nanometer
rpm	Rotations *per* minute
V	Volt
kV	Kilovolt

GENERAL STRUCTURE OF THE DISSERTATION

1. GENERAL STRUCTURE OF THE DISSERTATION

This dissertation is divided into five main parts:

PART I – Introduction and aims of the dissertation

This part contains an introduction to the topics that have been studied in this dissertation. Each topic is covered in a general way, with the exception of those which are most important for the interpretation of the results. The main goals of this dissertation are listed at the end of this part.

PART II – Experimental section

This part provides information regarding sampling and the experimental procedures used in the evaluation of the parameters under study.

PART III – Results and discussion

This part presents the results of the different studies and attempts to correlate them with the existing works addressing similar issues.

PART IV – Conclusions

The conclusions reached with this dissertation are summarized in this part.

PART V – References

This last part contains the literature required for the elaboration of this dissertation.

PART I

INTRODUCTION

AIMS OF THE DISSERTATION

2. INTRODUCTION

2.1. Generalities

Facing the oceans and its fauna and flora as a source of bioactive metabolites has revolutionized the scientific world and brighten a new hope to the scientific community. Marine organisms have shown to constitute a rich source of bioactive compounds with a diversified use in several areas, such as agriculture, food, textile and pharmaceutical industries. They have been intensively studied in the last few decades because of their promising bioactive molecules, perceived by the scientific community as good candidates for the development of new and innovative drugs (1). Marine organisms produce a large amount of secondary metabolites that are not found in terrestrial ones. In part, due to the extreme environmental conditions to which they are exposed, both marine animals and algae have developed defense strategies that resulted in a significant level of structural and chemical diversity of compounds. These compounds are originated from different metabolic pathways and are structurally different from those produced by terrestrial plants (2). The exploitation of these organisms for pharmaceutical purposes has revealed important chemical prototypes for the discovery of new agents, stimulating compounds isolation and syntheses of new related compounds with biomedical application.

Recent surveys of drugs from natural sources have shown that algae are promising organisms that provide new biochemically active compounds, with beneficial effects on human health and nutrition. Chemical research on algae products has been intensified during the last years and over 15000 primary and secondary metabolites from different metabolic pathways were reported (1). Scientists have focused their attention on secondary metabolism, which leads to a high structural diversity of compounds, due to modifications and combinations of reactions from the primary metabolic pathways. Nevertheless, both types of metabolites are of extreme importance and can be related to remarkable positive effects on human organism. In addition to bioactive compounds isolation, more recently, algae farming and controlled algae cultivation with the objective of producing valuable new compounds in a large scale has gained ground (3).

The bioactivity of seaweeds' extracts and isolated compounds constituted a great deal of research in recent years. This increasing interest is justified by the promising biological activities of their compounds, from both metabolic and pharmacological points of view. In order to valorize the Portuguese algal flora and extend its application for food and pharmaceutical industries, this dissertation will focus the study of bioactive compounds from seaweeds, and their role in health promotion and disease prevention.

2.2. Seaweeds

Algae are considered a big group of autotrophic organisms, ranging from unicellular to multicellular forms. These organisms can be found in both marine and fresh waters, and are divided according to their size in microalgae and macroalgae or seaweeds (4). Microalgae constitute a polyphyletic group of prokaryotic and eukaryotic microscopic organisms with a simple cellular structure. This group of unicellular microorganisms live individually or in groups and is known as phytoplankton. Among the most common microalgae are the Cyanophyceae (blue green algae), Bacillariophyceae (diatoms) and Chrysophyceae (golden algae). Among these, diatoms are the dominant life form in phytoplankton and represent the largest group of biomass producers on earth (5).

Macroalgae can be defined as photosynthetic multicellular eukaryotic organisms, with a wide variety of cell morphologies and life cycles. These organisms are found in a multiple variety of habitats and their morphological diversity results from their polyphyletic origin within the eukaryotic tree of life. In their natural environment, macroalgae grow on rocky substrates and form stable, multi-layered, perennial vegetation (6). A wide range of specific requirements, such as nutrients, salinity, temperature, light, depth and currents, are determinant factors that affect macroalgae growth and nutrients production. Nevertheless, the fact of being fixed on a substrate enhances macroalgae growth and productivity, contrary to what happens with phytoplankton (6, 7). These organisms are of extreme importance as primary producers; they play a key role in the productivity of oceans and constitute the basis of the marine food chain. Additionally to the organic material they produce through sun light, carbon dioxide (CO_2) and water (H_2O), macroalgae are one of the major responsible for oxygen production on earth (8).

Several classifications have been proposed over the years (8). Nowadays, macroalgae are divided into three main groups according to the distribution and occurrence of several photosynthetic pigments, which are the main features for algae classification. As so, they are normally divided into green algae (Phylum Chlorophyta), red algae (Phylum Rhodophyta) and brown algae (Phylum Heterokontophyta, Class Phaeophyceae). With the exception of the green and red algae that belong to kingdom Plantae, brown algae belong to the kingdom Chromista (9, 10).

Both “macroalgae” and “seaweed” terminology have been used when referring to these organisms, although the second respects to macroalgae living in marine waters. Along this dissertation, the term “macroalgae” will be used in a generalized context and

the term “seaweed” will be confined to macroalgae species exclusive or collected in marine waters.

2.2.1. Chlorophyta

Chlorophyta are considered to be the ancient of terrestrial plants and, due to their similarities with them, they are grouped in the kingdom Plantae (11). This phylum is better represented in fresh water (90%) than in the sea (10%) and counts more than 7000 known species (12, 13). Contrary to fresh water species, which have a cosmopolitan distribution, Chlorophyta species belonging to the marine environment from northern and southern hemispheres present clear differences (14).

In this phylum, the chloroplasts contain chlorophyll *a* and *b*, β-carotene and xanthophylls in a proportion resembling terrestrial plants. Lutein is the main carotenoid. Their major photosynthetic pigment, responsible for their green color, is chlorophyll *b*, although, some tropical species are pigmented by siphonoxanthin and siphonein (15). Concerning storage products obtained from photosynthesis, Chlorophyta differs from other eukaryotic macroalgae since they reserve starch in organelles called pyrenoids, within chloroplasts, instead of cytoplasm. The cell walls of these organisms are composed by cellulose as the major polysaccharide, although xylans and manans can replace cellulose in some species (14).

Among the full diversity of green algae, species belonging to the Ulvaceae family are specially disseminated (10). Ulvaceae species can tolerate a wide range of temperatures and survive to drying at low tide. *Ulva lactuca* Linnaeus is an edible macroalgae commonly found in Portuguese waters. It growths attached to rocks, crustaceous or other algae species (**Figure 1 A**), without a stipe, in the middle to low intertidal zone and has the appearance of a lettuce, thus being called sea lettuce or green laver. This species has a delicate and translucent thallus sheet like and its color varies from dark to light green (**Figure 1 B and C**). This opportunistic algae is capable of rapid colonization and growth when conditions are favorable. It has a fast growth rate and capacity to store and respond to enhanced nutrient supply (16). It is mainly composed by sugars and proteins, being commonly consumed in salads or used in soups (17).

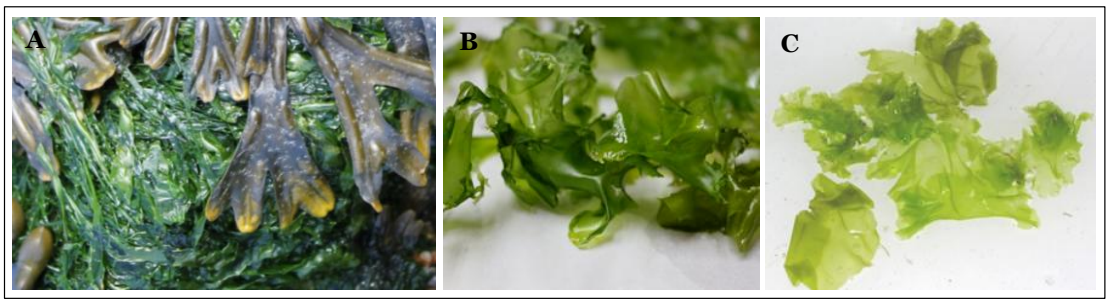

Figure 1. *Ulva lactuca* growing on rocks and attached to other seaweeds species (**A**); Color variations among *U. lactuca* (**B** and **C**). *(Photographs of Graciliana Lopes).*

2.2.2. Rhodophyta

Rhodophyta, one of the oldest eukaryotic algae, are thought to be descendent from a cyanome in the Glaucophyta. More than 6000 species have been identified to date, of which only a few are found in fresh water (10, 18). Rhodophyta can occur at all latitudes, but predominantly from equator to colder seas. Few Rhodophyta are found in polar and sub-polar regions, where green and brown algae are predominant, contrary to what happens in temperate and tropical regions where this phylum prevails. Members from this phylum share a combination of characters that do not occur together in any other eukaryote, such as the complete lack of flagellated stages and the absence of centrioles (14).

These species contain a variety of pigments, including chlorophyll *a*, phycobiliproteins, red phycoerythrin, blue phycocyanin, carotenes, lutein and zeaxanthin. The most important pigment is phycoerythrin, which provides the algae's red pigmentation by reflecting red light and absorbing blue light. Red algae with little of this pigment may appear more green or blue than red due to the presence of other pigments. Unlike other algae phyla, and thanks to their photosynthetic pigments that absorb light in the wavelength corresponding to blue color, red algae have the ability to live below 200 m depth in the ocean (19). These photosynthetic reserves are stored in plastids as floridean starch grains (14).

Several species from this phylum are important food crops, particularly in Asian countries, where members of the genus *Porphyria* are highly consumed (20). In Portugal, algae are consumed in trace quantities, the consumption of red algae being almost limited to the species *Osmundea pinnatifida* (Hudson) Stackhouse, also known as pepper grass, particularly in Azores Islands (21).

One of the most popular red seaweed species in Portuguese coasts is certainly *Asparagopsis armata* Harvey. Despite being native of southern hemisphere, this species is now globally distributed; in the north-eastern Europe it is more noticeable between June and September. *A. armata* appears as a pale purplish-red species, irregularly branched (with short branches alternating with longer ones) and with harpoon-like barbs (**Figure 2 A**) (21). This endemic species has a great capacity for growth and invasion, colonizing the habitat in which it lies and hindering the development of other species, such as algae and marine animals (**Figure 2 B**) (22).

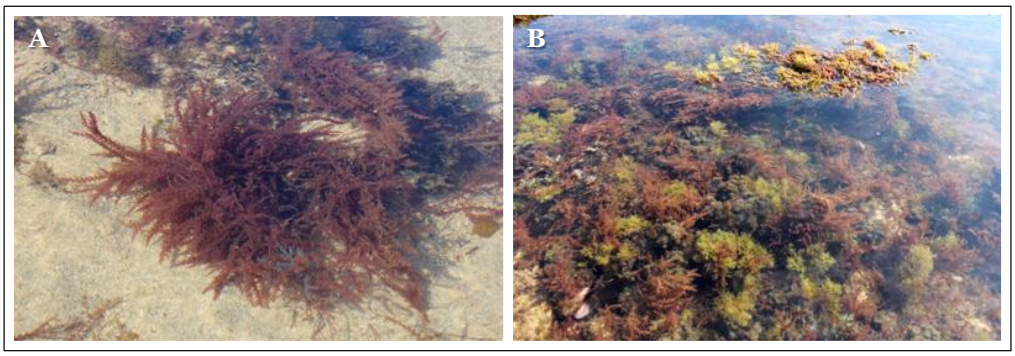

Figure 2. *Asparagopsis armata* (**A**) as invasive species (**B**). *(Photographs of Graciliana Lopes).*

2.2.3. Phaeophyta

Brown algae belong to the phylum Heterokontophyta (12) or Phaeophyta (23), according to the taxonomic classifications. As a matter of standardization, brown algae will be referred as Phaeophyta along this dissertation.

Phaeophyta mainly consists of macroscopic organisms inhabiting marine waters. Of the approximately 2000 known species, less than 1% colonizes fresh water habitats (24). These species have adapted to a great variety of marine ecological niches, including the tidal zone, rock pools, all intertidal zone and relatively deep waters near the coast. A large number of Phaeophyta are found in the intertidal or upper littoral area, where the waters are predominantly cool and the currents of nutrient-filled cold waters inflow the land (14). Species belonging to this phylum present a wide range of sizes and forms, varying from groups of threadlike cells with few centimeters to giant kelps with dozens of meters. These species grow so profusely that they form offshore kelp forests, very efficient at capturing sunlight and serving as home to a diverse group of marine animals. In a general way, brown algae species are composed by a holdfast (root like structure) (**Figure 3 A**), thallus (twig), stipe, lamina and pneumatocyst (also known as gas bladder, a floating structure

containing gas). Pneumatocysts are found only in brown algae and exert an upward force that allows the organism to receive more sunlight for photosynthesis (**Figure 3 B**) (25).

Figure 3. Brown algae species *Saccorhiza polyschides* (**A**) and *Fucus* (**B**). Evidencing the holdfast (**A**) and pneumatocysts (**B**). *(Photographs of Graciliana Lopes).*

Members of Phaeophyta are distinguished by their brown or yellow brown color, resulting from their main carotenoid fucoxanthin. Along with fucoxanthin, the main photosynthetic pigments of brown algae are chlorophylls *a*, *c*1 and *c*2, β-carotene, violaxanthin and diatoxanthin. Their brown color is also influenced by the presence of phlorotannins stored in vesicles called physodes (26). Contrary to Chlorophyta and Rhodophyta, their main photosynthetic storage product is not starch but laminarin, a water soluble β-(1→3)-glucan. Their cell walls are composed of cellulose, alginic acid and sulfated polysaccharides, produced in high quantities in some marine species and harvested for commercial purposes (27).

In fact, some Phaeophyta species are of sufficient commercial importance and have become subjects of extensive research. The alginates of some brown algae are harvested for commercial use as thickening agents in the textile, cosmetic and food industries. For many years macroalgae were also the source of iodine, a trace nutrient required in thyroid disorders (28). Despite the interest of the pharmaceutical industry regarding the search for new bioactive compounds, brown seaweeds are particularly attractive for cosmetics, where their bioactive ingredients show benefits beyond the traditional cosmetics, such as creams, lotions and ointments (29). Nowadays, the use of brown seaweeds does not have much visibility in Portugal. However, a few decades ago, the harvesting of seaweeds stirred the local economy. The "sargaço", also known as "argaço" or "limos", a set of diverse seaweeds (predominantly consisting of brown species from the genus *Saccorhiza*, *Laminaria* and *Fucus*, and also some red ones from the genus *Codium*, *Palmaria*,

Chondrus and *Gelidium*) growing on the cliffs of the Portuguese continental shelf was harvested and used to fertilize the soil and to improve its structure and capacity to increase H_2O retention (30).

2.3. Chemical composition

Since ancient times, seaweeds play an important role in Asian population diet, where they are also widely used by food industries because of their rich chemical composition and high content of fiber, minerals, vitamins and different antioxidants (**Figure 4**) (31). H_2O makes up 70 to 90% of the weight of fresh seaweeds. The composition of the dry seaweed varies with the species, but the approximate proportions are 45-75% of carbohydrates and fiber, 7-35% proteins and less than 5% fats. The main sugars are mannitol in brown seaweeds, sorbitol in red and saccharose in green. The primary mineral components are iodine, calcium (Ca^{2+}), phosphorous, magnesium, iron, sodium, potassium and chlorine. Concerning vitamins, seaweeds do not have vitamin D, but are rich in vitamin A, vitamins from B complex (B1, B2, B3, B6 and B12) and vitamin C. Taurin is an important amino acid present in seaweeds, as it is essential for the formation of bile salts (32).

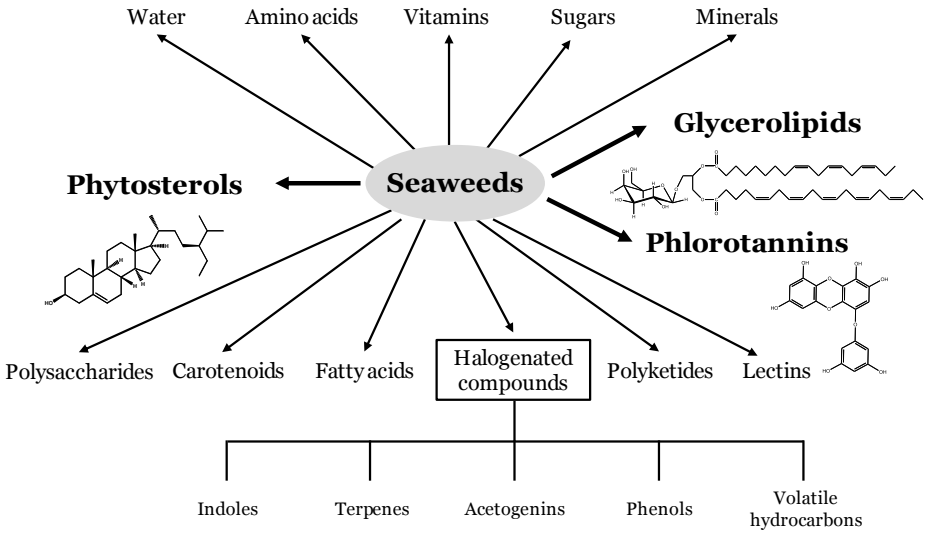

Figure 4. Main classes of compounds found in seaweeds with highlight on those that were studied in this dissertation.

Some of the secondary metabolites of seaweeds arose further attention, not only for their commercial application, but also for their biological activities and interesting health effects (**Figure 4**). Among them, polysaccharides like agar, carrageenans and alginate can be highlighted, as they are, by far, the seaweeds most economically important compounds.

These polysaccharides of high molecular weight are the main structural components of seaweeds cell walls and, despite some biological activities have been reported, they are important because of their extensive use in food and cosmetic industries, thanks to their gelling, viscosifying and emulsifying properties (33).

Of great importance are also carotenoids. These 40-carbon atoms structures are essential constituents of the photosynthetic apparatus, being responsible for light harvesting during photosynthesis. They also act as protein stabilizers and antioxidants. Carotenoids consist of a carbon skeleton derived from the polymerization of isoprene units that may undergo cyclization in one or both ends. The formed ring can be additionally substituted by oxo, hydroxyl (OH) or epoxy groups, originating different xanthophylls (34, 35).

Fatty acids are essential for cell function and have entered the biomedical and nutraceutical areas as result of their biological roles in clinical conditions, such as obesity and cardiovascular diseases (36). These compounds consist of a carboxyl group linked to a saturated or unsaturated aliphatic chain and play key roles in cellular and tissue metabolism, membrane fluidity, electron and oxygen transport and thermal adaptation. Seaweeds are rich in long chain polyunsaturated fatty acids, which are considered to be the most benefic for human health (37).

Halogenated compounds, mainly produced by red and brown seaweeds, comprise a vast and heterogeneous group of molecules dispersed in several different classes of primary and secondary metabolites, including indoles, terpenes, acetogenins, phenols and volatile hydrocarbons. The halogens present in these compounds are mainly bromine and chlorine and the antibacterial and antitumor activities are those of most pharmacological interest (38).

Polyketides and lectins are also interesting secondary metabolites present in seaweeds: polyketides, like macrolides, for their antibiotic application and lectins for their carbohydrate binding specificity, useful in immunological and histochemical studies (39, 40).

Mostly because of their rich and diverse chemical composition, seaweeds importance increased in such a way that in the past few decades the emphasis has moved from wild harvest to farming and controlled cultivation, in order to produce valuable and profitable new products in a large scale (41). This dissertation highlights the seaweeds composition in terms of phytosterols, phlorotannins and glycerolipids, and explores the biological activities of the most promising compounds.

2.3.1. Phytosterols

Phytosterols constitute a class of cholesterol-like molecules that are not endogenously synthesized by humans and are mainly found in the cellular membranes of plants and algae (**Figure 5**). These essential constituents of all eukaryotic membranes play important functions in the control of membrane fluidity and permeability and also in signal transduction, as hormones or hormonal precursors (42, 43). Beyond that, phytosterols represent a unique group that can be used as a chemotaxonomic biomarker for distinguishing members of the three main algal phyla (44).

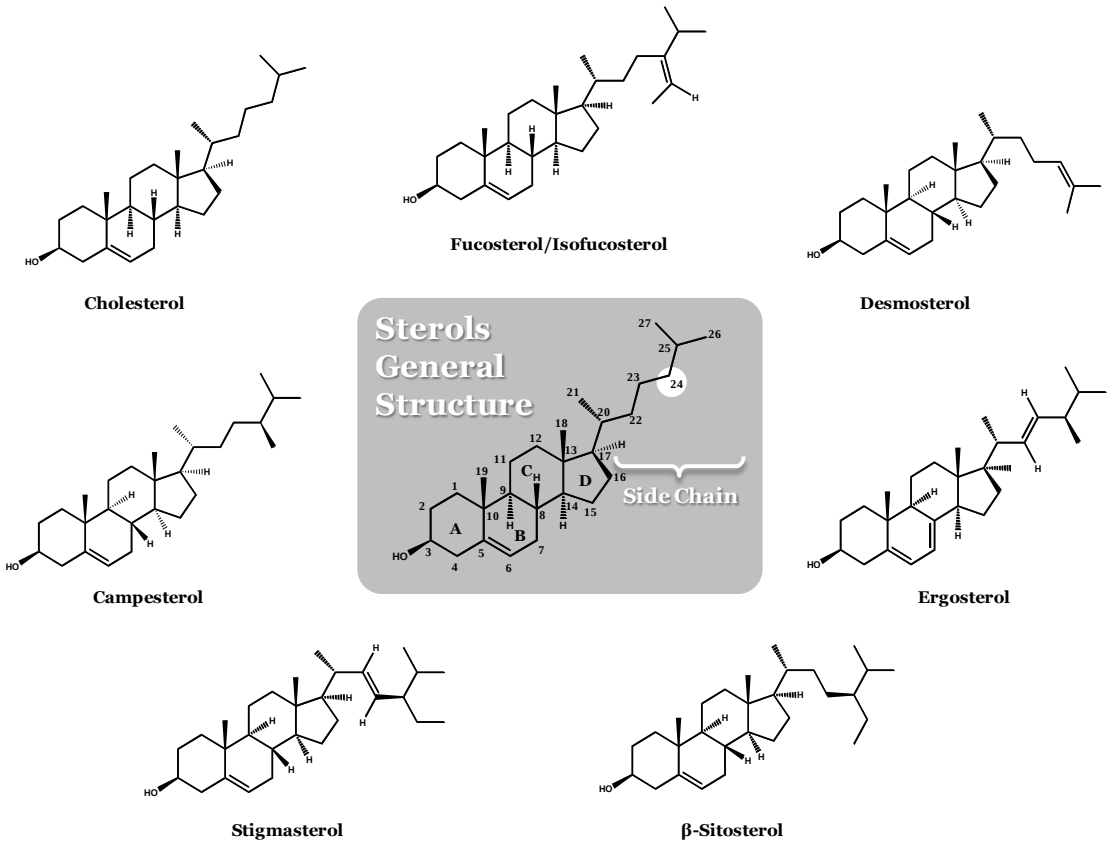

Figure 5: Phytosterols basic structure and chemical structures of the most common sterols found in seaweeds; * isomers at C-24: isofucosterol (*Z*) and fucosterol (*E*) (*adapted from (44)*).

Phytosterols are structurally similar and functionally analogous to cholesterol of vertebrate animals, differing only in the substitution on the sterol side chain, at the C-24 position. Therefore, whereas the cholesterol side chain comprises eight carbon atoms, most phytosterols side chains consist of nine or ten carbons (**Figure 5**) (42). The sterol nucleus contains four rings (A, B, C and D), OH group at C-3 and a side chain. Rings A and D are of particular importance for sterols function. The OH group at C-3 of ring A contributes for hydrogen-bond interactions. On the other hand, the conformation and

length of the side chain and the stereochemistry of the C-24 alkyl group in ring D are crucial for intermolecular interactions (**Figure 5**) (45). The alkyl substitution at C-24 in phytosterols is added by the enzyme sterol methyltransferase (SMT), which is not necessary in cholesterol and in the other C_{27} sterols biosynthesis. The activity of SMT controls the levels of cholesterol in photosynthetic organisms (46). Seaweeds phytosterols can be found either in their free form or conjugated with different molecules, like fatty acids and sugars and, in this situation, the OH group on the A ring is covalently bound to another molecule (44). Although it is more common to find algae phytosterols esterified with fatty acids, they can also appear conjugated with sugars by a 1-*O*-β-glycosidic bond between the 3-OH group and a hexose (commonly glucose) (47).

Sterols pattern tend to be quite stable among different algae groups; nevertheless, the ecological differences, geographical origin and developmental stage of the organisms can contribute to different phytosterols profiles (48). In a general way, C_{29} sterols, namely fucosterol and isofucosterol (**Figure 5**), are the major compounds in brown and green algae, respectively. Red algae, on the other hand, are richer in cholesterol. All of them possess β-sitosterol, campesterol and stigmasterol (**Figure 5**), which are the most frequent phytosterols in nature and in human diet (43).

The complexity of phytosterols is further increased by steric features concerning the size and direction of the substituent at C-24, which is a phylogenetic indicator. 24-β-Methyl sterols predominate in primitive organisms, while 24-α-ethyl sterols are typical of more advanced ones (49). Seaweeds belonging to Chlorophyceae division present more complex sterols with an asymmetric carbon at C-24, similar to the ones found in higher plants. Moreover, the proportion of C_{29} sterols in this group is high, in comparison with C_{27} and C_{28}, which supports green seaweeds as the ancestors of land plants (11).

2.3.1.1. Biosynthesis

Isoprenoids represent one of the most diverse families of compounds, some of them used in pharmaceutical and food industries for their considerable health benefits. As plants, algae utilize two distinct and separate compartmentalized pathways to build up the basic building blocks of isoprenoids, which take place in different cellular compartments: the cytosol and the plastids (**Figure 6**) (50).

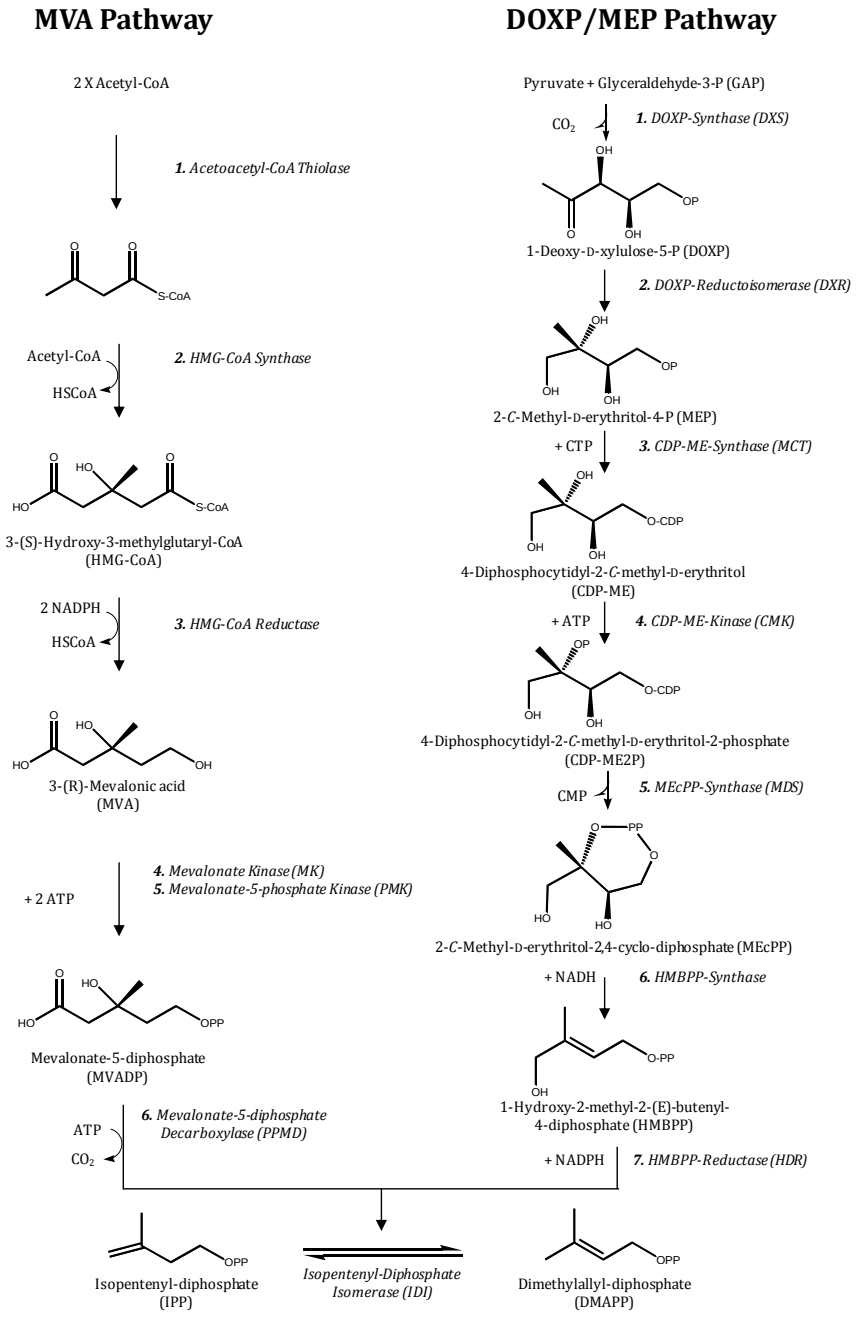

Figure 6. MVA and the DOXP/MEP pathways overview (44)

All isoprenoids biosynthesis starts with two basic units of five carbons: the isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). In the classical cytosolic pathway, known as mevalonate (MVA) pathway, IPP is formed from the condensation of three molecules of acetyl-coenzyme A (Ac-CoA). On the other hand, on the second non-MVA pathway for IPP/DMAPP biosynthesis, operating in the chloroplasts, IPP/DMAPP are formed from the intermediates pyruvate and D-glyceraldehyde-3-phosphate (GAP), to yield 1-deoxy-D-xylulose-5-phosphate (DOXP), followed by

intramolecular rearrangement and reduction to 2-*C*-methyl-D-erythritol-4-phosphate (MEP) (44). With the exception of green algae, which are devoid of MVA pathway and exclusively use the DOXP/MEP one for sterol formation, all photosynthetic organisms possess both biosynthetic pathways (51).

MVA Pathway

The MVA-dependent IPP/DMAPP biosynthesis takes place in the cytosolic compartment of algae cells and starts with the condensation of two units of Ac-CoA into acetoacetyl-CoA *via* a Claisen-type reaction, in which acetoacetyl-CoA thiolase acts as catalyst. Then, acetoacetyl-CoA and Ac-CoA condense to form 3-(*S*)-hydroxy-3-methylglutaryl-CoA (HMG-CoA), by the action of 3-hydroxy-3-methylglutaryl coenzyme A synthase. HMG-CoA is then converted into 3-(*R*)-mevalonate through a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reductive deacylation, catalyzed by HMG-CoA reductase. The remaining steps for IPP biosynthesis comprise two phosphorylation reactions to convert MVA to mevalonate-5-diphosphate (MVADP), catalysed by mevalonate kinase (MK) and phosphomevalonate kinase (PMK), and followed by an adenosine triphosphate (ATP)-dependent MVADP decarboxylation catalysed by mevalonate-5-diphosphate-decarboxylase (PPMD). Finally, isopentenyl-diphosphate isomerase (IDI) catalyses the formation of DMAPP, which is the chemically active isoprene unit that starts isoprenoid biossynthesis (44).

DOXP/MEP Pathway

The DOXP/MEP alternative pathway is initiated by a transketolase catalysed condensation of GAP with pyruvate. The enzyme responsible for this condensation, 1-deoxy-D-xylulose-5-phosphate synthase (DXS), yields the first product, DOXP. Subsequently, DOXP is transformed into the intermediate MEP in two steps catalysed by the DOXP reductoisomerase (DXR, also known as MEP synthase). An intramolecular rearrangement leads thereby to the formation of the enzyme-bound intermediate MEP, which is concomitantly reduced *via* a NADPH-dependent reduction. MEP is then converted into 4-diphosphocytidyl-2-*C*-methyl-D-erythritol (CDP-ME), which is phosphorylated yielding 4-diphosphocytidyl-2-*C*-methyl-D-erythritol-2-phosphate (CDP-MEP). These conversions are carried out by CDP-ME synthase, also referred to as MEP cytidyl transferase (MCT) and CDP-ME kinase (CMK), respectively. Next, CDP-MEP is converted into 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate (MEcPP) by ME-cPP synthase (MDS). This compound is then reduced by 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate synthase (HMBPP synthase) to yield 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-

diphosphate (HMBPP). In the final step, HMBPP is converted by the HMBPP reductase, HDR, into IPP and DMAPP. IPP and DMAPP become the basic building blocks of polyisoprenoid biosynthesis in the stroma of plastids. All MEP-derived isoprenoids are considered as typical and exclusive plastidial isoprenoids (44).

2.3.1.2. Sterols in seaweeds

The possibility of several biosynthetic pathways for sterols byosinthesis and the variability concerning the side chain provide a wide range of structural types of sterols in algae, which has long been recognized. These structures also provide strong evidence for particular phylogenetic affiliations in some algae taxa, since, unlike higher plants, 24-β alkyl sterols are dominant in algae. It is clear that sterols can play an important role in determining the relationships between algae and in their evolutionary relationships with other organisms. Thus, most of the algal divisions can be easily characterized on the basis of their sterol composition (52).

Rhodophyta is the unique phylum containing primarily cholesterol or related C_{27} sterols. They also produce small amounts of C_{28} and C_{29} sterols, C_{28} ones normaly being present in higher quantities (53). Desmosterol, the biosynthetic precursor of cholesterol, can also be found in red algae; nevertheless, it predominates in the evolutionary lower red algae (54). Some evolutionary higher red algae that are able to alkylate sterols contain 24-methylenecholesterol, which is a precursor of C-24 alkylated sterols. These sterols, together with the ones with a double bond at C-22, are found in low concentrations (55).

Green algae appear to be more variable in terms of sterols, suggesting some phylogenetic relationships (56, 57). In lower green algae, the alkylation of 24-methylenecholesterol leads to the production of an isomer of fucosterol, called isofucosterol. On the other hand, in the evolutionary advanced green algae and in higher plants, alkylation is followed by a reduction of the C-24 double bond, giving origin to the accumulation of sitosterol (54). Thus, in a general way, green algae have media percentages of C_{29} sterols that are high relative to their C_{27} and C_{28} sterols. In all green algae, sterols relative abundance follows the order $C_{27} < C_{28} < C_{29}$, while in all red algae, this pattern is reversed (57).

Brown algae present a sterols pattern different from the reds and more constant than the greens. This phylum almost exclusively contains fucosterol, which is biosynthesized through the alkylation of 24-methylenecholesterol. The fucosterol biosynthetic precursor, desmosterol, can be also present in brown algae, but usually at low concentration. Comparing with Rhodophyta, the desmosterol amounts in this phylum are

significantly lower. Generally, brown algae mainly contain C_{29} sterols and very low concentrations of phytosterols with less than 29 carbon atoms, presenting a high fucosterol:cholesterol ratio (54, 58).

2.3.1.3. Sterols bioactivities

Phytosterols are increasingly regarded as beneficial to human health, in such a way that they are also being included in several pharmaceutical formulations. The cholesterol lowering capacity of phytosterols is perhaps their main and most important property. Several studies have been performed to evaluate the extent of diet's phytosterols absorption (59, 60) and the cholesterol lowering properties displayed by them (61-66). In fact, phytosterols can lead to an additional significant reduction of serum cholesterol concentrations compared to that obtained with a low fat diet alone (47). These compounds have the ability to incorporate into membranes and to replace cholesterol molecules, inhibiting its absorption and attenuating the high-cholesterol diet effects (64). They primarily act at the intestinal lumen, where they compete with cholesterol for the incorporation in bile salts and phospholipid micelles. The competition of phytosterols with cholesterol for the uptake into intestinal epithelium leads to a reduction in cholesterol absorption by the intestinal cells, resulting from a displacement of cholesterol from the phospholipidic micelles (67). The recirculation of biliary cholesterol is another way of cholesterol absorption in the intestine, which can be reduced by phytosterols, once they can prevent the esterification of free cholesterol into cholesterol esters and, thus, its assembly into the chylomicrons (68).

Together with their cholesterol-lowering properties, phytosterols are also recognized as important molecules for the reduction of low density lipoproteins (LDL)-cholesterol and cardiovascular heart disease (CHD). Phytosterols have been shown to reduce LDL-cholesterol without relevant effects on high density lipoproteins (HDL) and triglycerides (68). Elevated LDL blood cholesterol is one recognized risk factor for CHD, an important cause of mortality and morbidity. Lowering LDL-cholesterol by dietary intervention is beneficial to human health, as it has been shown to reduce the risk of CHD. Untill now, there are no human studies demonstrating that plant sterols reduce the risk of CHD. However, both epidemiological studies and randomized controlled clinical trials have indicated a causal relationship between elevated LDL-cholesterol and CHD (69-71). To support this, the Eupean Food Safety Authority claims that there is evidence that the risk of CHD is reduced by cholesterol-lowering therapy, including dietary intervention strategies (72).

Apart from these activities, phytosterols also have an important role in reducing atherosclerosis, which increases with increasing levels of LDL-cholesterol, being reduced with increasing levels of HDL-cholesterol (73). They also display anticancer properties against human cancer cell lines, suggesting a protective effect of these compounds against colon, prostate and breast cancer (74, 75). According to all these studies, there is evidence for the exploitation of phytosterols rich matrices, in which seaweeds are included.

2.3.1.4. Extraction and purification

As mentioned above, algae sterols can occur is their free form, esterified with fatty acids or, in minor concentrations, involved in glycosylated conjugates. For this reason, the analysis of algae phytosterols requires not only the extraction of free and conjugated phytosterols, but also the hydrolysis of phytosterols conjugates, in order to convert the esterified phytosterols to their free form (47).

Sterols are usually extracted from algae material by solid/liquid extraction. Several solvents and solvent mixtures can be used in order to establish the most suitable extraction conditions (chloroform-methanol (MeOH), hexane, methylene chloride and acetone have been reported, among others) (76). After extraction, a saponification step (usually with ethanolic potassium hydroxide (KOH)) should be performed to enable the cleavage of ester bonds of phytosterol conjugates. Nevertheless, a combination of acid and alkaline hydrolysis is necessary to determine the total amount of phytosterols in the sample (including free, esterified, and glycosylated forms). After saponification, the free sterols can be removed from the extract by using solid-phase extraction (SPE) or simply by extraction with an organic solvent (normally n-hexane or diethyl ether) (77). To obtain the free purified compounds, the fraction containing sterols can be cleaned-up by preparative thin-layer chromatography (TLC) and column chromatography (CC), usually using silica gel stationary phases (78).

The main drawback of the sterols purification step is that it involves extensive and time/solvent-consuming procedures before phytosterols determination, which leads to a massive analysis time and also a significant lost of compounds (78).

2.3.1.5. Characterization by high-performance liquid chromatography (HPLC) with diode array detection (DAD)

HPLC coupled to DAD is a valuable tool for the separation of phytosterols, providing good repeatability and efficiency, short analysis time and reduced consumption of mobile phase (79).

Column packing and mobile phase

Separation by HPLC is performed in columns packed with microparticles of diameter comprised between 3 and 10 μm, called stationary phase. Columns packaging is achieved with high-pressure to ensure their stability during use. These smaller particles provide a higher surface of contact with the mobile phase, where individual components of the sample are moved. Because of the strong packaging of the stationary phase, the solvent (mobile phase) must be forced to flow through the column by a pump. The process of retention of the analytes is determined by the choice of the column packing and the selection of the mobile phase to push the analytes through the packed column. For this reason, the correct selection of the column packing and the mobile phase are the most important factor for a successful separation. The stationary phase can be of two types: normal or reverse. In reversed phase, the column packing is non-polar (normally C18), and the mobile phase solvents are H_2O and organic solvents miscible with H_2O (MeOH, acetonitrile). In normal phase, the column packing is polar (normally silica) and the mobile phase solvents are non-polar (hexane, isoctane, ethyl acetate (ETOAc)). In reverse phase columns, unlike the normal phase, polar compounds elute more quickly than non polar ones. This mode is used in more than 90% of the analysis (80).

For the separation of similar compounds in the samples there is a need for the use of mobile phases with solvent mixtures or systems in gradient. There are two basic methods for separation of compounds in HPLC, according to the composition of the mobile phase: isocratic and gradient. In isocratic mode, the mobile phase solvent composition remains constant with time, being the best mode for simple separations. In the gradient mode, the amount of one of the solvents increases with the time, being the best choice for the analysis of complex samples and unknown mixtures. Although gradients are adjusted according to the analytes, linear gradients are more common (81).

Thus, the separation is based on the affinity of the different compounds between the stationary phase and the mobile phase. The analysis of phytosterols is normally performed in reversed-phase columns (C18), and using an isocratic mode with solvent mixtures of MeOH:acetonotrile. As non-polar compounds, phytosterols attach to the column, and the elution is held according to the analytes polarity. Therefore, the more non-polar phytosterols are retained in the column for longer time (80, 81).

Detection

The universal detector for this type of compounds is the ultraviolet (UV). Because it is not destructive, it constitutes an advantage when there is the need to use another

subsequent detection system to acquire more information, or when there is the intention to isolate the compounds. The availability of DAD increases the possibility of identifying compounds as it registers the chromatograms at different wavelengths with a single injection: this detector provides the UV spectrum of each compound eluted, together with its retention time, which are two important parameters in the process of identifying compounds. The identification of phytosterols is possible by comparison of their retention time and UV spectrum with the retention times and spectra of the respective standards. In terms of quantification of each individual phytosterol, the most desirable is the relation to the area of the peak chromatographically obtained. However, the lack of reference substances, which allow the definition of the absorption characteristics of the compound, is a limiting factor (80, 82).

2.3.2. Phlorotannins

Phlorotannins are marine algae polyphenols with a wide range of molecular sizes. Although they have typically 10-100 kDa, their range can span from as small as 126 Da to as large as 650 kDa. Phlorotannins are also thought to polymerize as they age and thereby, go larger (83). These compounds are specific from brown seaweeds and have been reported from almost all brown algae orders (84).

These secondary metabolites are formed by the polymerization of phloroglucinol (1,3,5-trihydroxybenzene) units (**Figure 7**).

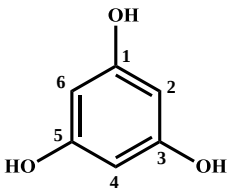

Figure 7. Phloroglucinol (1, 3, 5 – trihydroxybenzene).

Six different phlorotannins groups were found until now, based upon variations in their assemblage from the polymerization of phloroglucinol and the distribution of OH groups: phlorethols, fuhalols, fucols, fucophlorethols, eckols and carmalols (**Figure 8**) (85).

Phlorotannins with ether linkages: This subclass comprises phlorethols (**Figure 8 A**) and fuhalols (**Figure 8 B**), in which phloroglucinol monomers are linked by aryl ether bonds. Fuhalols differ from phlorethols because in this subclass phloroglucinol units are connected by aryl ether bonds in a regular sequence of *para-* and *ortho-* ether bridges.

Fuhalols are also characterized by the presence of one additional OH group in every third ring and by the lack of one or more OH groups in the whole molecule (86).

Phlorotannins with phenyl linkages: This subclass comprises fucols (**Figure 8 C**), which consist of phloroglucinol units linked by aryl-aryl bonds (86).

Phlorotannins with ether and phenyl linkages: This subclass comprises fucophlorethols (**Figure 8 D**), which contain both aryl-ether and aryl-aryl bonds (86).

Phlorotannins with dibenzodioxin linkages: This subclass comprises eckols (**Figure 8 E**) and carmalols (**Figure 8 F**). Eckols are usually of low molecular size. This subclass is composed of molecules with a dibenzodioxin unit substituted by a phenoxyl group at C4. Carmalols have a dibenzodioxin unit and are derived from phlorethols. Both subclasse can present additional OH groups (86).

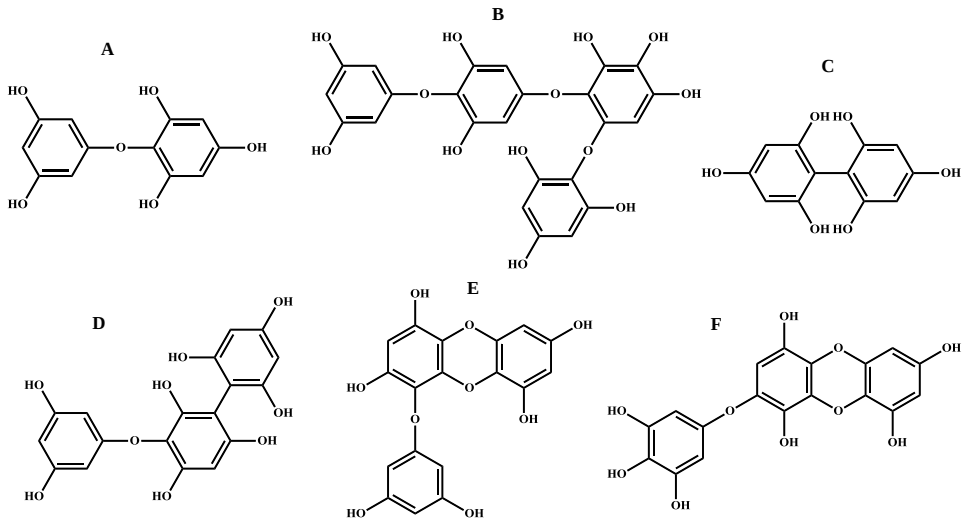

Figure 8. Phlorotannins groups structures: phloretol (**A**), fuhalol (**B**) , fucol (**C**), fucophloretol (**D**), eckol (**E**) and carmalol (**F**).

2.3.2.1. Biosynthesis

The exact biosynthetic pathway by which phloroglucinol gives origin to phlorotannins is not yet fully known, in part because of the difficulty in establishing methodologies to monitor phlorotannins synthesis at genetic and enzymatic levels (87). Nevertheless, it is known that the aromatic precursor of phlorotannins is biosynthesised by the acetate-malonate pathway, also known as polyketide pathway, in a process involving polyketide synthases-type enzyme complexes (**Figure 9**) (83, 84, 88).

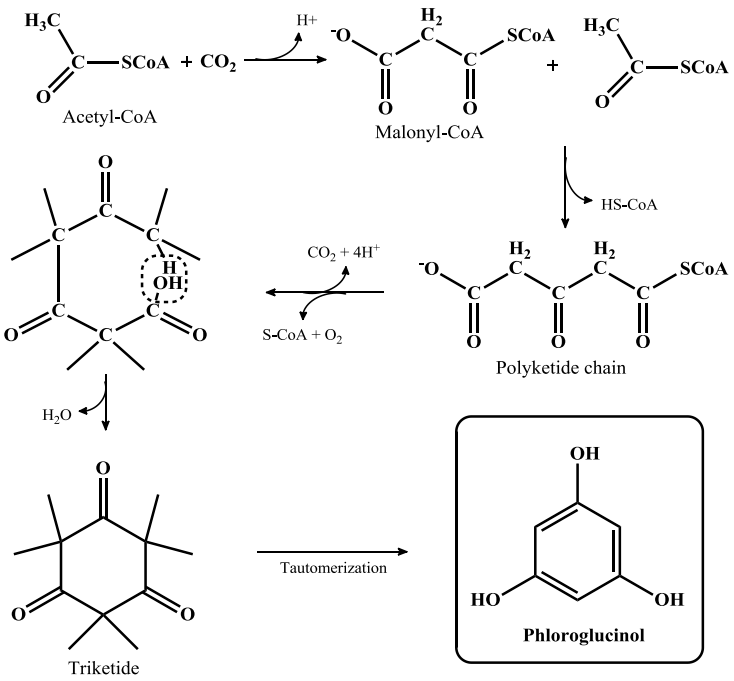

Figure 9. Phlorotannins biosynthetic pathway (*adapted from* (89)).

In the early steps of phlorotannin biosynthesis, Ac-CoA is converted into malonyl CoA through the addition of CO_2. This changes the acetyl methyl group into a highly reactive methylene, leading to a polymerization process, which occurs without a high investment of energy. During further synthesis steps, the CO_2, which was added as an activator, is lost (**Figure 9**) (89).

As a result of the polymerization process, a polyketide chain consisting of an acid moiety is formed, and the co-enzyme is lost. The polyketide chain is transformed by intermolecular ring closure and elimination of H_2O, producing a hexacyclic ring. The cyclization product is a triketide, which is not stable and, thus, undergoes transformation (tautomerisation) into its thermodynamically more stable aromatic form, phloroglucinol, consisting of three phenolic OH groups (**Figure 9**)(89).

2.3.2.2. Role in seaweeds

Phlorotannins are accumulated among the vegetative cells of the outer cortical layer of brown algae, regardless of the variety of tissue, stage of growth or organ (90). These compounds are stored in physodes, which can be recognized in meristodermic and promeristematic cells. Physodes accumulate at the zygote periphery early in development, being secreted into the primary zygote wall. These phenolic compounds are thought to prevent multiple fertilizations, by inhibiting spermatozoid movement, as they are also found on the surface of recently fertilized zygotes (91, 92). Nevertheless, in some species of

brown algae, phlorotannins tend to be concentrated within the outer cortical layers and the mitotic meristematic and meiotic sporogeneous tissues (84). Physodes containing phlorotannins can also contribute to the development of the algae cell walls. When physodes fuse with the cell membrane, and the phlorotannins are secreted from cells, they complex with alginic acid by means of hydrophobic, hydrogen, ionic and covalent bonds (92, 93). Despite the multifunction of phlorotannins in algae, their herbivore deterrent capacity and cell wall and antioxidant roles should undoubtedly be highlighted. For this, the location of these compounds is of central importance. Due to their peripheral localization they can absorb at short wavelengths and display a photoprotective role against UV radiation, attenuating the inhibition of photosynthesis and deoxyribonucleic acid (DNA) damage, which are two major detrimental effects of UV radiation (94).

For some years it was thought that brown algae could release phlorotannins to the sea water (84, 91), under the stimuli of external and environmental factors. Nevertheless, exudation of phloroglucinol and phlorotannins was confirmed to take place only after algae cell death. Hereupon, whilst the algae are alive, polymeric phlorotannins are strictly kept within the algal body, not being released into the sea water (95).

Several biological activities have been attributed to phlorotannins over the years. The UV protection against photo-oxidative stress (96), antioxidant (97-103), antimicrobial (102, 104-109), anti-allergic (110) and anti-inflammatory (111-114) are among the most important ones.

Of these, only the antimicrobial, anti-inflammatory and anti-allergic/anti-aging activities will be explored along this dissertation.

2.3.2.3. Extraction and purification

The procedures used for phlorotannins extraction are widely variable and include aqueous mixtures of ethanol and acetone. Acetone is believed to inhibit the interactions between phlorotannins and proteins during extraction and also to break hydrogen bonds between phlorotannin-protein complexes, increasing the total yield of extraction (84, 115, 116). Phenolic compounds are usually easily soluble in solvents less polar than H_2O and, in a general way, the efficiency of the solvent increases with increasing polarity (117). The most suitable solvent for phlorotannins extraction was studied by Koivikko and co-workers, who found the mixture acetone: H_2O (7:3) as the most efficient one. This research group also evaluated the number of consecutive solvent treatments in phlorotannins extraction and described that more than 80% of these compounds are released during the first treatment and that four treatments allowed the release of more

than 90% of soluble phlorotannins present in brown algae powdered samples. On the other hand, for the release of cell wall bound phlorotannins it is necessary to subject the sample to an alkaline degradation (118). As free phlorotannins are prone to rapid oxidation, in studies involving isolated compounds, acetylation with acetic anhydride-pyridine is used to protect them from oxidation (86). The conversion of peracetylated phlorotannins into their free form can be achieved by reductive ester cleavage, using lithium aluminium hydride with tetrahydrofuran as solvent (119). The addition of ascorbic acid has also been used to prevent oxidation of phlorotannins extracts, as it increases the stability of these compounds (120).

In order to obtain a phlorotannins rich extract, the mixture resulting from acetone:H_2O extraction can be purified, taking advantage of the phlorotannins features. As these polyphenols have a great adherence capacity to cellulose, the cellulose powder can be used to selectively remove phlorotannins from solution. The different affinities of these compounds to the solvent and the cellulose will allow their separation and subsequent purification from the other extracted compounds (121).

2.3.2.4. Quantitative analysis of phlorotannins

Since brown algae are known to contain no other phenolics than phlorotannins (84), colorimetric methods for total phenolics quantification seem to be a reliable tool. Nevertheless, the colorimetric quantification of these polyphenols has some limitations, as there is no selective method to degrade phlorotannins into its basic unit, phloroglucinol (84, 87, 121). Additionally, the colorimetric methodologies for phlorotannins quantification measure reactive phenolics, which correspond to the extractable physode-bound phlorotannins. Contrary to what happens with the cell wall-bound phlorotannins, the ones in physodes are reactive and they possess the characteristics of effective polyphenolic defences. On the other hand, phlorotannins which have been deposited at the cell wall, oxidized by peroxidases and complexed with alginic acid can not be extractable or detectable by these commonly used colorimetric assays (122).

The basis of colorimetric methods for polyphenols measurement relies on the quantification of total phenolic OH groups present in an extract, independently of the molecules in which they occur. Additionally, the chemical reactions in colorimetric assays do not always follow the stechiometry and the yield obtained represents an approximation of the compounds group under investigation (123).

Dimethoxybenzaldehyde (DMBA) assay

The DMBA assay is specific to quantify phenolic compounds substituted at positions 1,3 and 1,3,5. The reagent 2,4-dimethoxybenzaldehyde reacts with the OH groups at these specific positions and originates a pink colored product, which can be spectrophotometrically measured. This methodology is the most appropriated for phlorotannins colorimetric quantification, since these polyphenols are hydroxylated at positions 1, 3 and 5. Phloroglucinol is used as the monomer standard for phlorotannins quantification (87). The main advantage of this method is its insensitivity to interference; for example, it does not react with tannic acid containing only *ortho* and *para* hydroxyl-substituted phenolics (124).

In the reaction between DMBA reagent and phlorotannins there is an electrophilic attack by the aldehyde in a strongly acidic solution. The intensity of the color produced depends, in part, on the structure of the aldehyde formed, and the variation in reactivity depends on the differences in the chemical structures of phlorotannins. The main disadvantage of this assay is that it can form chromophores with some non-polar metabolites; nevertheless, this situation can be avoided if the colorimetric quantification is performed with purified fractions of phlorotannins (124, 125).

2.3.2.5. Characterization by liquid chromatography-mass spectrometry (LC-MS)

The availability of standards in the market allows the characterization of compounds present in a sample by comparison of their chemical characteristics with those of the respective standards. Thus, compounds which absorb in the UV-Vis are easily identified, after chromatographic separation, by comparing their retention time and absorption spectrum with the respective standard. Nevertheless, despite phloroglucinol, to date no phlorotannins standards are available. This constitutes a limitation to the identification of these molecules, as they exhibit different retention times and UV spectrum, depending on the degree of polymerization (which increases with age) and the nature of connections their basic units establish between each other. Some approaches to separate phlorotannins by HPLC have been performed in the past few years; nevertheless, the studies were focused on a phlorotannins profile without identifying or quantifying the individual compounds (120, 126, 127). For these reasons, HPLC-DAD is far from ideal for characterizing these compounds and more specific methods are required to separate, identify and quantify individual phlorotannins, in order to make possible the assignment of specific biological activities and pharmacological effects to specific compounds.

LC-MS is an advantageous technique, which combines the chromatographic separation by liquid chromatography with the detection by mass spectrometry (MS). In a general way, the molecules present on the sample are converted into a gas phase ionic species by the addition or removal of electrons or protons (**Figure 10**) (128).

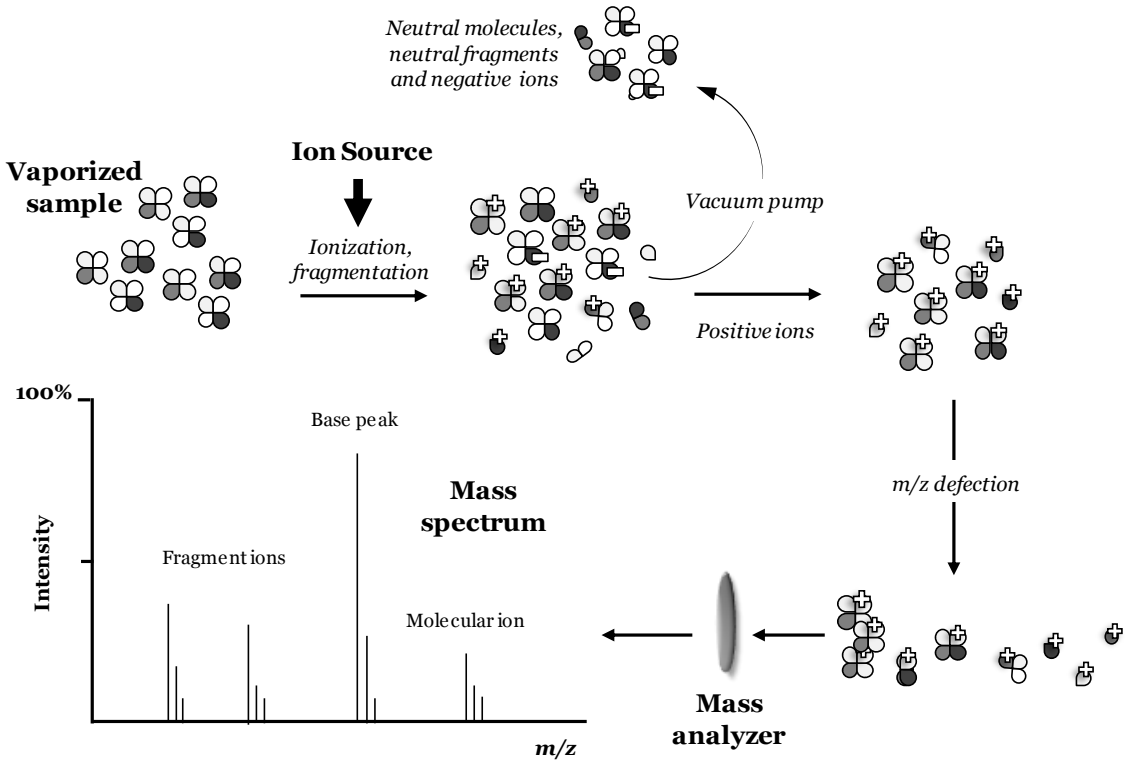

Figure 10. Schematic representation of a sample analysis by MS, operating in positive ion mode.

These ionic species consist of atoms, molecules or fragments of molecules carrying one or more positive or negative electrical charges. One of the most common ion formation techniques consists in bombarding the gas phase sample with a beam of electrons. During this procedure, an electron (e^-) is removed from the highest occupied molecular orbit of the molecule (M), forming a positively charged molecular ion (**equation 1**) (128).

$$M + e^- \rightarrow M^{+\bullet} + 2e^- \quad \textbf{(1)}$$

Nevertheless, depending on the mode of ionization, a variety of ion types can be formed, for instance:

$[M+H]^+$ - positively charged ion/protonated molecule;

$[M-H]^-$ - negatively charged ion/deprotonated molecule;

$[M]^{\bullet+}$ - positive radical ion, formed by the removal of an electron from a molecule;

$[M]^-$ - negative molecular ion, formed by the addition of an electron to a molecule;

$[M+Na]^+$, $[M+Cl]^-$, $[M+NH_4]^+$- adduct ions, formed by the addition of an ionizing species to a molecule.

The molecular ions are separated in the mass analyzer, according to their mass-to-charge (*m/z*) ratio (**Figure 10**). The separated ions are then passed to the detector system, which measures their concentration, and the result is finally displayed in the form of a mass spectrum (128). Since the ions on the gas phase are very reactive (susceptible to collisions and interactions) and short living, their formation and manipulation normally occurs under high vacuum (129).

Thus, despite the vacuum system (responsible for maintaining the low pressure in the mass spectrometer) the detector (which stores, processes and displays the data) and the inlet system (responsible for transferring the sample into the ion source, HPLC and gas chromatography (GC) being the most common), a mass spectrometer comprises fundamentally two important components:

1. Ion source - where the samples are ionized prior to their analysis in the mass spectrometer. There are a variety of ionization techniques, which convert the neutral molecules into gas-phase ions. The ion sources can be a liquid-phase or solid-state. In the liquid-phase, the analyte is introduced by nebulization into the source where ions are produced at atmospheric pressure and directed to the mass spectrometer through some vacuum pumping stages. Electron spray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo-ionization (APPI) correspond to this type. In the solid state ion sources, the analyte is in the form of a deposit, which is then irradiated by energic particles or photons that desorb ions from the surface of the deposit. Of this is example the matrix-assisted laser desorption ionization (MALDY). Generally, the ion source is kept at atmospheric pressure and continuous pressure and voltage gradients are used from the source to the detector in order to pump out the ions through the analyzer (128-130).

2. Mass analyzer – this component is considered the heart of the mass spectrometer and is responsible for the separation and analysis of the ionic species according to *m/z*. As examples of mass analyzers are the magnetic/electric sector, the linear quadrupole ion trap (LIT), three-dimensional quadrupole ion trap (QIT), the orbitrap, the time of flight (TOF) and cyclotron resonance (ICR); they can be selected according to their resolution, mass range, scan rate and detection limits (129).

ESI

ESI consists in a soft ionization technique to ionize intact chemical species by multiple charging. Thus, a very little residual energy is retained by the analyte and few or no fragmentation occurs (131). ESI is produced by the application of a strong electric field (under atmospheric pressure), over a solution passing through a capillary tube, under a weak flux. A charge accumulation will be induced at the liquid surface allowing the formation of highly charged droplets. The droplets will pass through a steam of a heated inert gas, which removes the lasting solvent molecules. After solvent evaporation, the droplets shrink and their charge *per* unit volume increase (129, 132, 133).

The major advantage of ESI is the possibility to produce multiple charged ions from large molecules, which improves the sensitivity at the detector and allows the analysis of high molecular weight molecules, as is the case of phlorotannins (83, 129). Due to its characteristics, ESI shows a good response over analytes with ionizable acid/basic polar functional groups. Due to the advantages of this instrumentation and due to the limitations of DAD for the characterization of phlorotannins, HPLC has been coupled with ESI-MS for the molecular fractionation prior to MS analysis. HPLC-ESI/MS has become a very powerful technique capable of analyzing both small and large molecules of various polarities in complex biological samples (134).

Mass spectrum

In HPLC/LC-MS, data is presented in form of a chromatogram, a mass spectrum being displayed for each analytical signal. In a mass spectrum there is a molecular ion region, corresponding to the ion formed by the removal of one or more electrons, and a fragment ion region, where the molecule-related fragments can be found (**Figure 10**). Thus, the molecular mass of the analyte is often represented by the peak with the highest *m/z* value of the mass spectrum. This peak corresponds to the molecular ion ($[M]^{\bullet+}$) or to the protonated molecule ($[M+H]^+$). The base peak is the most intense peak in the spectrum and represents the most stable ion resulting from the ionization process. The intensities of all other ions are normalized in accordance to the base peak. Fragment ions appear with masses lower than the molecular mass and reflect the amount of energy added to the molecules during ionization (higher the energy, higher the decomposition of the molecular ion) (**Figure 10**). In a general way, the ions resulting from ESI are multiple charged and the analyte remains intact (135).

Mode of ionization

MS has traditionally deal with positively charged ions, this being the ions polarity more commonly formed. Nevertheless, instruments are designed to handle in both positive ion mode (PIM) and negative ion mode (NIM), according to the extent of protonation and deprotonation of the analytes. The extent of protonation and deprotonation of the molecules contained in the analyte depends on various factors, including the nature and structure of those molecules. Substances having basic groups, such as amines, amides and esters, are usually analyzed in PIM, since they are easily protonated. On the other hand, substances with acidic functions like carboxylic acids and phenols are easily deprotonated and, therefore, negatively analyzed. The nature of the solvent and pH are also important in generating positive and negative ions, as the charged droplets are formed by the solvent. When the solution containing the analyte is pumped through the capillar, an electrochemical reaction occurs and the solvent suffers oxidation (PIM) or reduction (NIM), which will supply positive or negative ions in the solution. The positive ions are analyzed in solvents with low pH, while negative ions are produced from solutions of chloroform and high pH values. In a general way, polar solvents like H_2O, MeOH and acetonitrile are commonly used in ESI because of their facility to undergo electrochemical reactions (136, 137).

As so, in ESI, the ionization can occur in PIM or NIM. In PIM, the spraying needle is maintained at positive potential and the charging occurs *via* protonation. In NIM, a negative potential is kept and charging occurs by deprotonation of the analyte. This makes the detected ionic species not corresponding to the true ion but the protonated or deprotonated molecules (138). The way to find the mass of the analyte by its mass spectrum is based on the *Mathematical Charge Deconvolution*, that extracts the molecular mass information from spectra showing sequences of peaks due to ions with varying number of charges, being nowadays part of ESI/MS instruments (131, 139, 140). Despite the foregoing, the ionization mode is commonly chosen according to the best molecular ionization.

The fragments and molecular ions obtained in a mass spectrum are specific of each compound and function as a compound fingerprint. Thus, concerning phlorotannins, it is common to find fragments with *m/z* corresponding to 126, which is the molecular weight of their basic unit phloroglucinol (126 g/mol). Moreover, by the *m/z* of the molecular ion it is possible to know how many molecules of phloroglucinol constitute the polymer. With a more detailed analysis, it is also possible to predict the kind of linkages established between the phloroglucinol units since, according to the chemical linkages, the molecules

will break and produce different kinds of fragments. Thus, this first analysis of the LC-MS data gives much more information about these compounds than the analysis of the data resulting from HPLC-DAD (141).

In the absence of commercially available standards, an effective way to obtain accurate information about a compound is through its isolation (142). Once the compound has been isolated and its structure has been assigned (by other methodologies of analysis, for instance, nuclear magnetic resonance (NMR), correlation spectroscopy (COSY) and heteronuclear multiple Quantum correlation (HMBC), it is possible to assign it a mass spectrum and to construct a library.

2.3.2.6. Compounds isolation

The isolation of compounds is in the basis of discovery, development and manufacture of drugs. For achieving it, and in order to obtain enough quantity for chemical characterization and structure elucidation, huge amounts of samples are necessary, together with a wide array of solvents with variable polarities. Diverse chromatographic techniques are in the basis of compounds isolation, the affinities between the stationary and mobile phase being critical for that.

Column chromatography

Column chromatography allows the separation of compounds from a mixture, for example, in crude extracts, or the isolation of products resulting from chemical reactions from synthesis. Column chromatography uses a stationary phase, normally packed in glass columns. The mixture of compounds is applied at the top of the column and the mobile phase is allowed to flow through the stationary phase. The mobile phase can flow through the stationary phase by gravity or with the application of positive pressure, which is used to force the eluent down the column at a faster rate; in this situation, the technique is called vacuum liquid chromatography (VLC). In column chromatography, each component of the mixture will exhibit different interactions with the stationary and mobile phases, which will depend on the chemical structure and geometry of each molecule. This allows each component of the mixture to be carried down the column at variable rates so that separation can be achieved (143).

Normal vs reversed phase chromatography

There are basically two types of solid/liquid column chromatography, the normal-phase (NP) chromatography and the reversed phase (RP) chromatography. In NP

chromatography, the stationary phase is more polar than the mobile phase. Several types of stationary phases can be used, silica gel being the most common. It provides optimum and reproducible compounds purification and is available in a variety of shapes and sizes (e.g. Silica Gel 60, which means a pore diameter of the silica particles of 60 Å). This polar stationary phase composed of silanol groups (**Figure 11 A**) allows the less polar molecules to elute first (as polar molecules experiment a greater degree of retention than non-polar ones) (143).

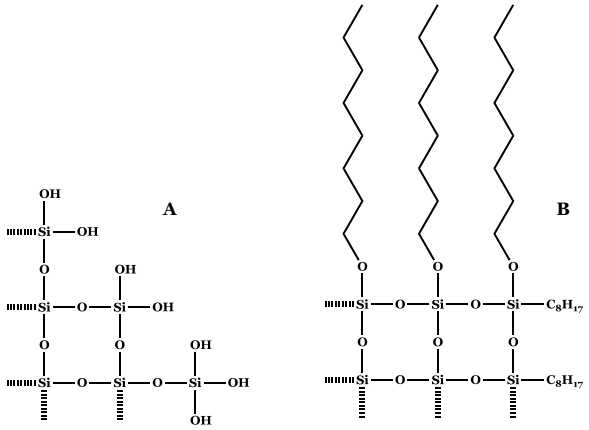

Figure 11. Schematic representation of silica used in normal phase (**A**) and reversed phase chromatography (**B**).

Contrarily, in RP chromatography the stationary phase is non-polar. The non-polar stationary phase can be produced by covering the polar surface of the silica gel with a non-polar layer of alkanes (**Figure 11 B**). The mobile phase is generally a binary mixture of H_2O and a miscible polar organic solvent, like MeOH, acetonitrile or tetrahydrofuran (143).

Compounds separation can be achieved in isocratic conditions, where the mobile phase is not changed during separation. On the other hand, a gradient separation is more commonly used and, in this situation, the composition of the mobile phase is changed during separation (143).

TLC

TLC is a chromatographic technic commonly used to monitor compounds separation during the isolation process. It is performed in a thin support, generally aluminum or glass, covered by a thin-layer of stationary phase, generally silica. The mixtures to be separated are applied in a spot at about 1 cm from the bottom of the TLC plate. The solvent is allowed to evaporate and the plate is placed on a transparent separation chamber containing the mobile phase. The mobile phase goes up the plate by capillary

action, allowing compounds separation. The separation is enhanced by varying the content of the mobile phase, which is performed according to the coating of the TLC plate (144).

After complete elution, the TLC place is analyzed. The components of the mixture are separated and give origin to spots with different retention factors (*Rf*). As the compounds present in the mixture can give origin to colorless spots, it is necessary the use of additional methods for their viewing. Thus, the spots can be visualized under UV light, at 254 nm. If they are not visible this way, the TLC plates can be revealed by spraying the plates with TLC visualization reagents. *p*-Anisaldehyde reagent is a preparation of *p*-anisaldehyde in glacial acetic acid and sulfuric acid. This reagent is sensitive to most functional groups, especially the strongly and weakly nucleophilic, and tendentiously insensitive to alkenes, alkynes and aromatic compounds. The revelation of the TLC sprayed with *p*-anisaldehyde reagent requires heating to 110 ºC. *p*-Anisaldehyde tends to stain the TLC plate itself to a light pink color, while other functional groups tend to vary with respect to coloration (144, 145).

2.3.3. Glycerolipids

Glyceroglycolipids are the most widespread group of lipids in nature. They are synthesized by all eukaryotic and prokaryotic organisms and constitute important membrane lipids with a key role in energy storage, membrane formation and fluidity and in chemical interactions with the environment (146, 147). This group consists of amphipathic molecules formed by the joint of fatty acids and glycerol by ester bonds. The glycerol molecule can be mono-, di- or tri-substituted by fatty acids, originating mono-, di- or triglycerides, respectively. Beyond fatty acids, the glycerol molecule can establish glycosidic linkages with one or more sugar residues, which, together with the fatty acids, originate glycosilglycerols, commonly found in plant membranes (147, 148) (**Figure 12**).

Of them, the ones with galactose in their composition constitute the main part of plant membranes, monogalactosyldiacylglycerols (MGDGs) and digalactosyldiacylglycerols (DGDGs) being the most common (148). Brown seaweeds play an important role in Asian countries' diets, in part because they contain valuable nutrients and bioactive components, some of which have not been found in terrestrial plants. Especially the species grown in temperate or subarctic areas can accumulate Ω-3 and Ω-6 polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (EPA, 20:5 Ω-3), stearidonic acid (SDA, 18:4 Ω-3) and α-linolenic acid (LNA, 18:3 Ω-3) being the major Ω-3 and arachidonic acid (ARA, C20:4 Ω-6) the major Ω-6 PUFA (149, 150).

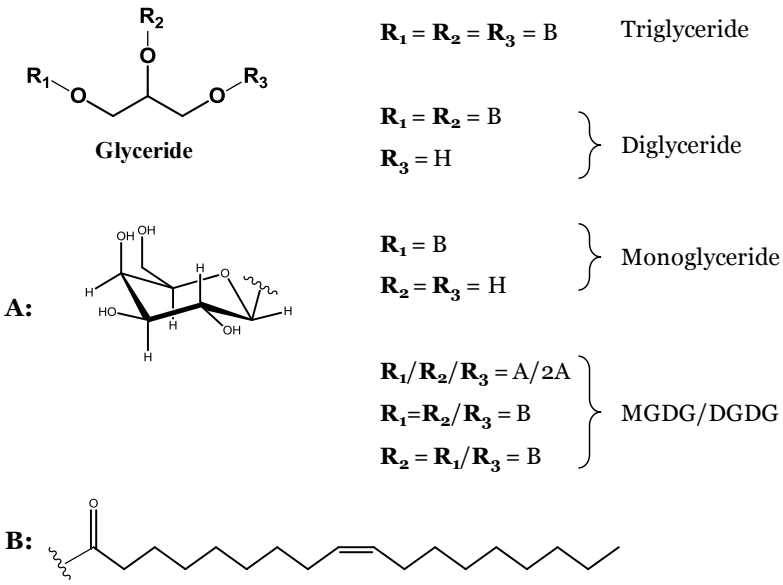

Figure 12. Structures of triglycerides, diglycerides, monoglycerides, MGDGs and DGDGs. Galactose – **A**, oleic acid – **B**.

Contrarily to what happens in animals and yeasts, glycoglycerolipids are the major membrane lipids of brown seaweeds, having a special importance in photosynthetic membranes (151, 152). The high contents of PUFAs in brown seaweed lipids are attributed to the high levels of these PUFAs in glycoglycerolipids, as they constitute the major brown seaweed lipids class (149). Most brown seaweed lipids have higher contents of Ω-3 PUFAs, which can constitute an advantage for the increment of the Ω-6/Ω-3 ratio, considering brown seaweeds incorporation into food products (153). Excessive amounts of Ω-6 PUFAs and a very high Ω-6/Ω-3 ratio is found in Western diets, which promotes the pathogenesis of many diseases, including cardiovascular disease, cancer and inflammatory and autoimmune diseases, whereas increased levels of Ω-3 PUFAs exert suppressive effects (154).

Only the anti-inflammatory activity of glycerolipids will be explored along this dissertation.

2.4. Biological activities

As referred above, there are numerous biological activities attributed to secondary metabolites from marine origin, where macroalgae hold a special place (155). The biological activities assigned to these organisms are related to both extracts and isolated compounds (107, 108). Among them, the antimicrobial, anti-inflammatory and anti-allergic activities are especially important, since there is a search for new biological compounds with potential to replace those used in therapeutics for which there are already many resistances (156, 157). The antioxidant activity is, by far, the most explored, in part because the oxidative stress is a condition present in most of the pathologies (158, 159).

2.4.1. Antioxidant activity

For its general importance and relationship with numerous pathologies, the antioxidant potential of crude and purified extracts or isolated compounds is assessed by almost all researchers in biological studies. As so, during the last decades, research on oxidative stress has been subject of a rapid progression and antioxidants have gained growing notoriety among the scientific community and general public (160).

2.4.1.1. Oxidative stress

Oxidative stress can be defined as a biological condition occurring from the imbalance between the production of reactive species and their detoxification through biological systems (enzymatic and non-enzymatic) that remove or repair the damage they cause. All living organisms have biological systems responsible for removing and repairing the damage caused by free radicals, existing a balance between them and the molecules responsible for their neutralization. Every time this redox balance is disturbed, and there is an increment in the production of reactive species, there will be damage in all cellular components, including proteins, lipids and DNA, resulting in a state of oxidative stress (161). Thus, the maintenance of the equilibrium between the production of reactive species and antioxidant defenses is an essential condition for normal organism functioning. Nevertheless, despite their association with several diseases and pathological conditions, such as neurological diseases and inflammatory states, and with the aging process, reactive species can be beneficial to the organism when used by the immune system to destroy the pathogens, or when acting as messenger molecules in cell signaling pathways (162, 163).

Oxidative stress may result from natural causes, like situations of extreme exercise or inflammation, and from non-natural causes, such as the presence of xenobiotics in the body (164). There is a continuous need for the inactivation of oxidants formed as a normal product of aerobic metabolism. The intake of dietary antioxidants may help to maintain an adequate antioxidant status. Moreover, it is recognized that antioxidants other than vitamins can constitute a major factor responsible for such protective effects (165).

2.4.1.2. Reactive species

There are several reactive species directly involved in the establishment of oxidative stress. Nevertheless, the most common are the reactive oxygen species (ROS) and the reactive nitrogen species (RNS) (166, 167).

Oxygen molecule has low reactivity; however, due to its electronic configuration, it can be activated or reduced, leading to the formation of toxic molecules (166). ROS include radical and non-radical species derived from oxygen and represent the most important class of reactive species generated in living systems. Among the radical species, hydroxyl ($^{\cdot}OH$), peroxyl ($ROO^{\cdot}$), alkoxyl ($RO^{\cdot}$) and superoxide anion ($O_2^{\cdot-}$) are the most representative. In turn, the non-radical ones include hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hypochlorous acid (HOCl) (167). $O_2^{\cdot-}$ is formed by reduction of triplet molecular oxygen ($^3O_2$), in a process mediated by enzymes, such as NADPH oxidases and xanthine oxidase (XO) or, non-enzymatically, by redox-reactive compounds like the semi-ubiquinone compound of the mitochondrial electron transport chain (**Figure 13**). Superoxide dismutase (SOD) is responsible for the conversion of superoxide into hydrogen peroxide (168). In biological tissues, $O_2^{\cdot-}$ can also be converted non-enzymatically into the non-radical species H_2O_2 and $^1O_2$ (169). In the presence of reduced transition metals, such as ferrous or cuprous ions, H_2O_2 can be converted into the highly reactive $^{\cdot}OH$, or into H_2O, by the enzymes catalase (CAT) or glutathione peroxidase (GPx) (**Figure 13**) (170). $O_2^{\cdot-}$ is the primary ROS produced in cells. Due to the various conversions that $O_2^{\cdot-}$ might be targeted, most of the effects observed in cellular and non-cellular systems are indeed not directly mediated by $O_2^{\cdot-}$, but rather by its derived ROS. Of those, the reactions with cellular lipids, proteins and DNA may be highlighted, since they can lead to cell damage and death (171).

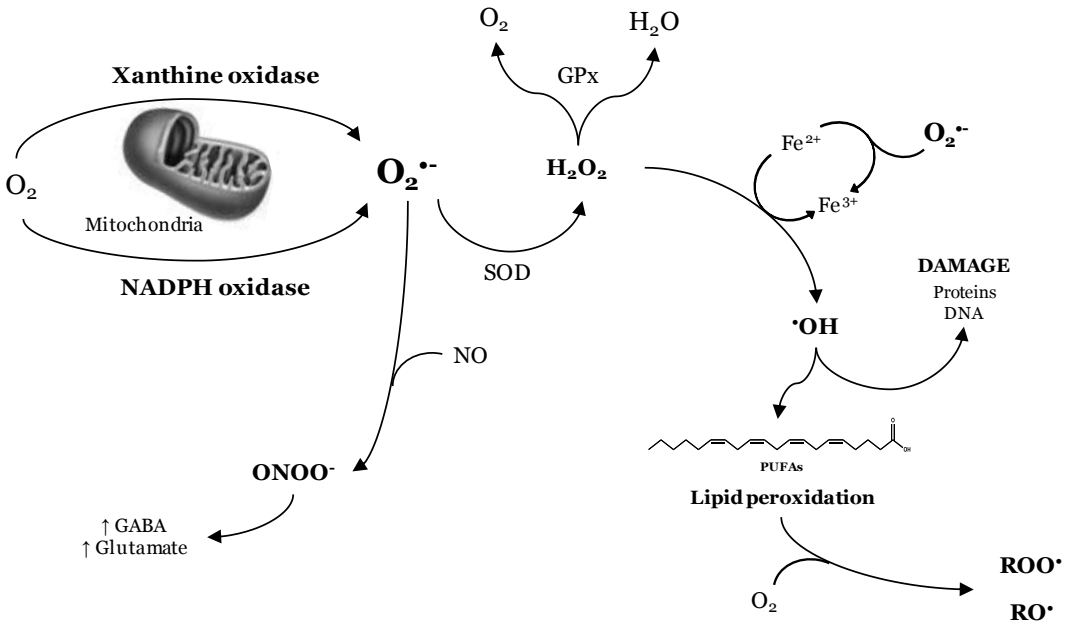

Figure 13. Schematic representation of the generation of several ROS and RNS. NADPH - nicotinamide adenine dinucleotide phosphate reduced form; $O_2^{\bullet-}$ - superoxide radical; SOD – superoxide dismutase; H_2O_2 – hydrogen peroxide; GPx – glutathione peroxidase; $^{\bullet}OH$ – hydroxyl radical; PUFAs – polyunsaturated fatty acids; $ROO^{\bullet}$ - peroxyl radicals; $RO^{\bullet}$ - alkoxyl radicals; NO – nitric oxide; $ONOO^-$ - peroxynitrite; GABA – ɣ-aminobutiric acid.

Among RNS, the nitric oxide radical ($^{\bullet}NO$) and the non-radical reactive species peroxynitrous acid (ONOOH) and peroxynitrite ($ONOO^-$) can be highlighted, the last ones being extremely harmful for the central nervous system (CNS) (172). $^{\bullet}NO$ is produced in higher organisms by the oxidation of L-arginine, in a process catalyzed by the enzyme nitric oxide synthase (NOS) (173, 174). Depending on the microenvironment, NO can be converted to various other RNS, such as nitrosonium cation (NO^+), nitroxyl anion (NO^-) or $ONOO^-$ (175). $^{\bullet}NO$ toxicity is predominantly linked to its ability to combine with superoxide anions (**Figure 13** and **equation 2**):

$$^{\bullet}NO + O_2^{\bullet-} \rightarrow ONOO^- \quad \textbf{(2)}$$

The half-time of $^{\bullet}NO$ lasts only a few seconds in an aqueous environment and its stability is higher under low oxygen concentrations. However, since it is soluble in both aqueous and lipid media, it readily diffuses through the cytoplasm and plasma membranes, affecting neuronal transmission in CNS. In extracellular medium, $^{\bullet}NO$ reacts with oxygen and H_2O to form nitrate and nitrite anions (176). When the generation of RNS in a system exceeds the system's ability to neutralize and eliminate them, a situation of nitrosative stress occurs.

The role of NO in biological systems and its relation with pathological states will be further explored in the section concerning inflammation.

2.4.1.3. Antioxidants

Antioxidants can be defined as substances that delay or prevent the oxidation of an oxidizable substrate (177). Defense against free radicals-induced oxidative stress involves preventive and repair mechanisms and physical and antioxidant defenses. The antioxidant defenses can be exogenous or endogenous and, within the last, enzymatic and non-enzymatic. Enzymatic antioxidant defenses include SOD, GPx and CAT, while non-enzymatic ones are represented by ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids, among other antioxidants. Under normal conditions, there is a balance between both the activities and the intracellular levels of these antioxidants, which is essential for organisms' homeostasis (177, 178). Antioxidants can act *in vivo* or *in vitro* by inhibiting the generation of reactive species, or by direct sequestration of these species. *In vivo*, an antioxidant can also act indirectly and increase the endogenous antioxidant defenses, by increasing the expression of genes encoding for SOD, CAT or GPx, which prevent the formation of reactive species. The various defenses complement each other and act on different oxidizing agents or cellular compartments (164).

2.4.1.4. Evaluation of the antioxidant potential

The evaluation of the antioxidant capacity of natural extracts or compounds can be easily evaluated by spectrophotometric methods. However, this screening does not reflect the physiological conditions, but constitutes a first approach to the biological potential of extracts and compounds (179).

Macroalgae have been deeply explored in the last decades and, apart from the isolation of compounds from different chemical groups, several kinds of extracts have been evaluated for their capacity to scavenge free radicals. Because macroalgae are photosynthetic organisms, they are exposed to sun's radiation and to high concentrations of oxygen, which promotes the formation of free radicals. The absence of structural damage in these organisms indicates a good protection against oxidation and, for this reason, these organisms are a good source of secondary metabolites with antioxidant potential.

The evaluation of the antioxidant activity of macroalgae extracts has been a constant over times (98, 99, 180, 181). Several kinds of crude extracts, purified extracts and isolated compounds have been screened in order to find the main compounds or groups of compounds responsible for the antioxidant activity in each phylum. Alongside the enormous antioxidant capacity of carotenoids, one of the main responsible for the

antioxidant activity attributed to brown macroalgae are polyphenols (100). This capacity has been proved by the *in vitro* evaluation of extracts and isolated compounds using human cell lines (101, 182). Recently, Parthiban and co-workers evaluated the antioxidant capacity of ethanolic and acetonic macroalgae extracts and verified that the antioxidant potential was directly proportional to the amount of polyphenols present in the extract (98). Since phlorotannins are the unique polyphenols of brown algae (84), the antioxidant activity of these organisms can be mainly attributed to these compounds. Fujii and colleagues (183) isolated phlorotannins from the brown algae *Eisenia bicyclis* (Kjellman) Setchell and reported these compounds as potent antioxidant agents, with eckol and fucofuroeckol A being the most active, presenting a stronger activity than the natural polyphenols kaempferol, quercetin, myricetin and chlorogenic acid (183). The phlorotannins eckstolonol (184) and fucodiphlorethol G (185) where reported as strong radical scavengers. Other compounds were reported in brown algae for their antioxidant activity, namely meroditerpenoids (186, 187) and monogalactosyldiacylglycerols (188).

2.4.2. Lipid peroxidation (LPO)

LPO is directly related to oxidative stress, since it consists in the oxidative degradation of lipids. Lipids are targets of many ROS and RNS and are oxidized to give a diverse array of products. Thus, *in vivo* LPO has been implicated as the underlying mechanism in numerous disorders and diseases, such as cardiovascular and neurological diseases and aging. Nevertheless, it is unclear if LPO is a cause or consequence of such pathological events (189). There are three basic mechanisms leading to the peroxidation of lipids, namely free radical-mediated oxidation, free radical-independent oxidation and non-enzymatic oxidation, and each mechanism requires specific antioxidants (190). Due to its etiology, only free radical-mediated oxidation will be discussed in this dissertation.

In free radicals-mediated LPO, one initiating free radical can oxidize many molecules of lipids, which occur in biological membranes and low density lipoproteins (191). Different types of reactive species can be involved in this process, namely $RO^{\cdot}$ and $^{\cdot}OH$ (which can arise from the Fenton reaction of H_2O_2 and Fe^{2+}, from peroxynitrite, or from high energy irradiation) (**Figure 14**).

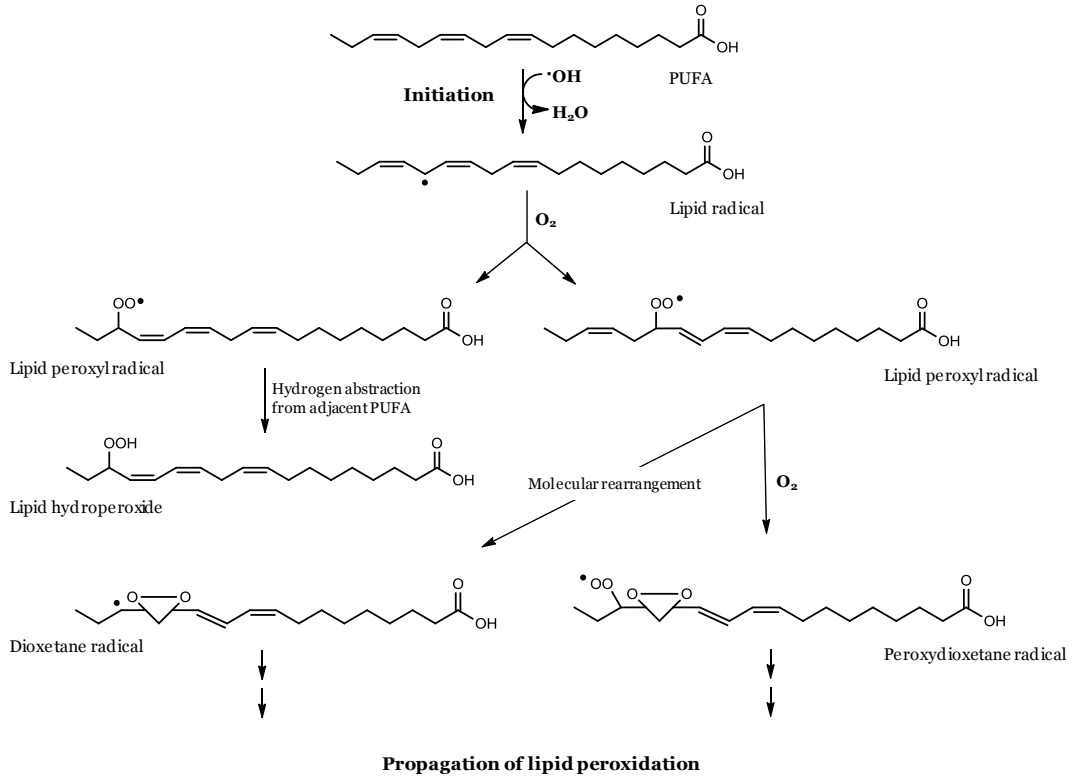

Figure 14. Schematic representation of free radical-mediated lipid peroxidation. PUFA – polyunsaturated fatty acid; ·OH – hydroxyl radical; H_2O – water; O_2 – oxygen.

LPO (**Figure 14**) is initiated by the abstraction of hydrogen from PUFA originating a carbon-centered radical. This radical can undergo molecular rearrangement to form a more stable compound. The addition of O_2 to the lipid radical originates a lipid peroxyl radical, which can form a hydroperoxide *via* the abstraction of hydrogen from the adjacent fatty acid, or suffer molecular rearrangement and addition of O_2 to form new radicals, leading to the propagation of LPO. Of these reactions, the important chain propagation step is the abstraction of bisallylic hydrogen from lipids by lipid peroxyl radicals to give conjugated diene lipid hydroperoxide and new lipid radicals, which continue another chain reaction (192).

2.4.2.1. LPO inhibition

Linoleic acid (LA) has been used to study the capacity of drugs to inhibit LPO, not only because it is the most abundant PUFA in living organisms, but also because its oxidation proceeds by a straightforward mechanism and yields simple reaction products, which does not always happen with other PUFAs (193).

The effect of high molecular weight phlorotannins on LPO was evaluated by Wei and co-workers in mouse liver, *in vivo*. These authors observed that these polyphenols strongly inhibited the generation of malondialdehyde (a product of LPO) (194).

Phlorotannins isolated from three Laminarians (eckol, phlorofucofuroeckol A, dieckol and 8,8′-bieckol) showed potent phosphoLPO inhibition in a liposome system (103). The LPO inhibition recorded in fish oil and LA was evaluated with an extract of *Hizikia fusiformis* (Harvey) Okamura and attributed to its polyphenolic compounds (195). In fact, some years before, the capacity of phlorotannins to prevent fish oil rancidity was evaluated by Xiaojun and colleagues, who proved that phlorotannins purified from a Sargassaceae presented an antioxidant capacity in this field 2.6 times higher than a 0.02% *tert*-butyl-4-hydroxytoluene (BHT) solution (196). These polyphenols have also shown capacity to prevent the LPO reaction induced by UV radiation, suggesting that phlorotannins play a role in intra and extracellular protection by absorption and oxidation processes (197, 198). Due to their role in health and tissue homeostasis, antioxidants and LPO have been also taken as biomarkers of tissue damage (199).

In addition to the health benefits provided by the inhibition of LPO, some researchers have mentioned that ROS and lipid oxidation in food industry can be controlled or minimized by the addition of commercial synthetic antioxidants or natural antioxidants (200).

2.4.3. NO and Inflammation

Inflammation can be defined as a mechanism of innate immunity by which the organism responds to harmful stimuli. As a complex biological response of vascular tissues, inflammation is mainly characterized by vasodilatation, which results, in a large extent, from NO-dependent processes(201, 202).

NO is highly permeable and diffuses rapidly across the membranes. Because it is responsible for promoting blood vessels relaxation, it was also termed as endothelium relaxing factor (201). In inflammatory responses this effector molecule can modulate the release of a wide range of inflammatory modulators, enzymes activity, blood flow and adhesion of leucocytes to the vascular endothelium (202). Under normal physiological conditions NO produces an anti-inflammatory effect. Anyway, once over-produced, it acts as a pro-inflammatory mediator and induces inflammation. Having the endothelial cells as the main target, this molecule leads to vasodilatation in cardiovascular system and is involved in immune responses by cytokine-activated macrophages (203). As a neurotransmitter, it acts at neuron synapses and regulates apoptosis (204). Thus, under pathological conditions, the undesirable effects resulting from the overproduction of this molecule are, in a general way, related to vasodilatation, inflammation and tissue damage. Face to this, molecules with the capacity to suppress the overproduction of NO and avoid

tissue destruction, have been seen as potential anti-inflammatory agents. Both inhibitors of NO biosynthesis and synthetic L-arginine analogous demonstrated a successful effect on this field (201, 205).

NO is produced through its precursor, L-arginine, by the action of the specific enzyme NOS. From the activity of NOS, by oxidation, also results the amino acid L-citrulline (173, 174) (**Figure 15**).

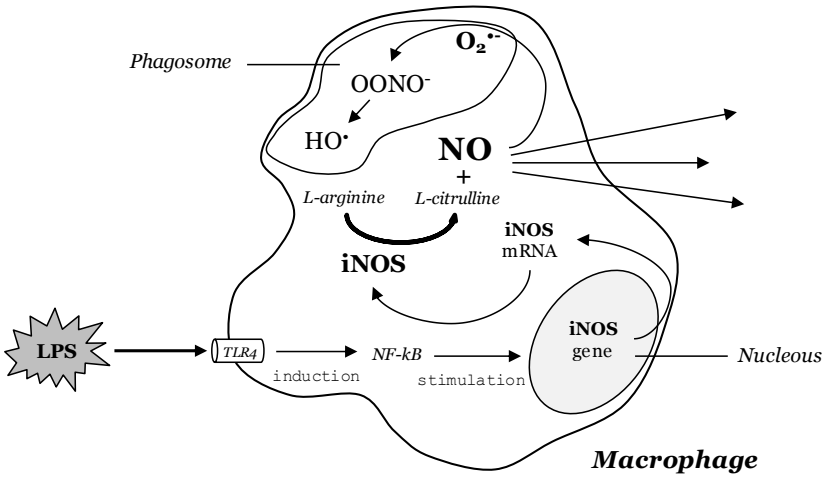

Figure 15. Schematic representation of NO production by iNOS in macrophages. LPS stimulation induces NF-kB, which stimulates the production of iNOS through the activation of iNOS gene in the nucleus. iNOS produces NO and L-citrulline through the precursor L-arginine. NO is released to the extracellular medium. In the phagosomes, NO interacts with $O_2^{\bullet-}$ originating $ONOO^-$ and $HO^\bullet$. LPS – lypopolysaccharide; TLR4 – toll like receptor 4; NF-kB –nuclear factor kappa; iNOS – inducible nitric oxide synthase; mRNA – messenger ribonucleic acid; $O_2^{\bullet-}$ - superoxide anion; $ONOO^-$ - peroxynitrite anion; $HO^\bullet$ - hydroxyl radical.

There are three isoforms of NOS, all of them composed by an oxygenase and a reductase domain (homologous with cytochrome P-450 reductase). Two of the three isoforms of NOS were first isolated from rat neurons and bovine endothelial cells and named neuronal NOS (NOS I) and endothelial NOS (NOS III), respectively. These enzymes are constitutively expressed in cells and responsible for synthesizing NO in response to an increase in Ca^{2+} or stress stimuli (**Figure 15**) (206, 207). The third isoform, the inducible NOS (iNOS or NOS II), is constitutively expressed in some tissues and is typically synthesized in response to inflammatory or pro-inflammatory mediators, such as cytokines or endotoxins (like bacteria products) (208).

Macrophages are a major component of the mononuclear phagocyte system and play a critical role in initiation, maintenance and resolution of inflammation (208). These cells

start the production of NO, for example, after exposure to bacterial lipopolysaccharide (LPS), and continue its production for several hours. The NO produced can combine with $O_2^{\bullet-}$ in the phagosomes, forming $OONO^-$ and consequently $^{\bullet}OH$, which is used by these cells to kill ingested bacteria (**Figure 15**) (209). The over-production of NO by iNOS can lead to septic shock, which is characterized by hypotension, inadequate tissue perfusion and organ failure, often resulting in death. Sepsis can be managed by drugs with the capacity to inhibit NO production or action (210).

2.4.3.1. Anti-inflammatory activity

The use of macrophage cell lines as a model for anti-inflammatory capacity evaluation is a common procedure. The ability of isolated compounds and extracts from a wide range of matrices to reduce the NO in the extracellular medium of LPS-stimulated macrophages is evaluated. The reduction of the NO in the culture medium is not enough, *per se*, for attributing an anti-inflammatory activity to a compound or extract. This happens because the decrease in NO levels can be done by simple scavenging. In order to attribute anti-inflammatory activity to any matrix, other complementary tests have to be performed, for instance, the evaluation of citrulline or the evaluation of the NO scavenging in a cell-free system.

In recent years, it has been paid growing attention to the anti-inflammatory activity of both extracts and isolated phlorotannins from seaweeds (211-215). Perhaps because of their wide distribution and ecological importance, species from the genus *Eisenia* and *Ecklonia* have been the most studied (216). Jung and co-workers isolated five phlorotannins from an *Eisenia* species (eckol, dieckol, 7-phloroeckol, phlorofucofuroeckol A and dioxinodehydroeckol) and found that they dose-dependently inhibited LPS-induced NO production at non-toxic concentrations (212). In another work, Sugiura and co-workers went further and studied the same seaweed species in an *in vivo* model for the first time (213). These authors tested four isolated phlorotannins and the results were as expected, with a good anti-inflammatory activity for the tested compounds (eckol, 8,8'-bieckol, phlorofucofuroeckol A and B). Kim and colleagues evaluated the antioxidant and anti-inflammatory activity of phlorotannins isolated from a species of *Ecklonia* and concluded that phlorofucofuroeckol A significantly inhibited the LPS-induced production of NO and PGE_2 through the down-regulation of iNOS and cyclooxygenase 2 protein expression (217). Other works performed with seaweeds extracts attribute their anti-inflammatory activity to these polyphenols (218, 219).

Along with phlorotannins, the anti-inflammatory activity of glycerolipids has been studied in different cell lines. These compounds have the ability to down-regulate iNOS expression in cells exposed to LPS, thus exerting an anti-inflammatory action by the reduction of NO production (220, 221). Some structure-activity relationships have been established for this kind of compounds. Although the mechanism of their anti-inflammatory action is not completely understood, it is known that glycerolipids with higher levels of unsaturation are more effective in inhibiting iNOS. On the same way, this action is also dependent on the position of the unsaturations (221).

2.4.4. Hyaluronidase (HAase)

Skin is the largest human organ and constitutes its first line of defense from external factors. As an interface with the environment, skin is particularly vulnerable to ROS, since it is exposed to oxidative stress from both endogenous and exogenous sources (222, 223). Although oxidative stress is a key factor in this process, hyaluronic acid (HA) also plays a significant role (223). This anionic, non-sulfated glycosaminoglycan forms the core of proteoglycan, which consists of heavily glycosylated proteins responsible for maintaining the proper volume and flexibility of the skin. The integrity of HA inside the dermal matrix is essential for cell integrity, mobility and proliferation (224). Under oxidative stress, HAase, the enzyme responsible for the depolymerization of HA, is over-activated and excessively breaks down HA, leading to the destruction of the proteoglycan network. This results in the deregulation of skin homeostasis and aggravates inflammatory and allergic states. Besides the easy spread of allergic and inflammatory mediators, occurring from the change in proteoglycan network, skin loses sustainability and gets aged appearance (224).

2.4.4.1. HAase inhibition

The inhibition of this enzyme can play an important role in several pathological states. Even though in an indirect way, HA has an important role in the propagation and exacerbation of allergic, inflammatory and infectious states. The prevention of HA's degradation *via* inhibition of HAase contributes to maintain the normal skin structure, as well as its barrier function, reducing the spread of propagation of allergens, ROS, RNS and microorganisms (225).

The effect of phlorotannins in preventing HA's degradation *via* HAase inhibition is related not only to the prevention of skin aging, but also to the reduction of inflammatory states, allergy and migration of cancer cells. These compounds can effectively contribute to the recovery of skin homeostasis and consequently prevent the downstream events that physically damage the dermal matrix structure. They are not only potent ROS scavengers,

but have also demonstrated a huge capacity to inhibit HAase and to minimize oxidative stress through a synergy created by the elimination of ROS and enhancement of the antioxidant defense capacity (110, 111, 226). This feature, along with the potent anti-aging activities, is the hallmark of phlorotannins that enables effective protection from the loss of elasticity of aged skin (224). For these reasons, the use of natural anti-aging products derived from marine sources is gaining prominence over the years (157).

2.4.5. Antimicrobial activity

2.4.5.1. Bacteria

Bacteria are considered the first inhabitants of earth and their number overcomes any other biological life form. These prokaryotic single celled microorganisms present a wide range of shapes (**Figure 16**) and can be found alone, or forming groups or arranged as filaments (**Figure 16**) (227). Bacteria live almost everywhere, from soil, water, waste, animals and plants to the deeper areas of earth (227, 228).

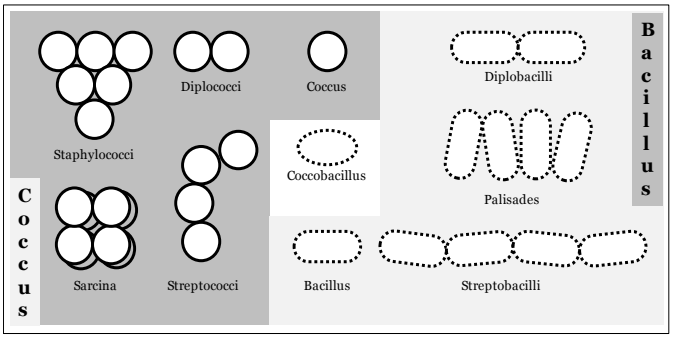

Figure 16. Simplified representation of different shapes and arrangements of bacteria.

Basically, bacteria can be divided into two main types, the Gram positive ($Gram^+$) and the Gram negative ($Gram^-$), according to their reaction to the Gram stain test. While $Gram^-$ have a peptidoglycan cell wall surrounded by a second layer of LPS and lipoproteins, $Gram^+$ only have a thin peptidoglycan cell wall. For this reason, and because of the presence of the LPS endotoxin, $Gram^-$ bacteria are more toxic than $Gram^+$ (227, 229). Regarding growth, these microorganisms have four growth phases: lag (slow growth and adaptation to the environment), log (exponential phase of growth until nutrients depletion), stationary (reduce cells metabolism due to depleted nutrients) and death. Both types of bacteria can be cultured in laboratory using solid culture media (agar plates), which are the most appropriated for cultures' isolation. The use of selective culture media is a common practice for bacteria isolation, since different strains have different

nutritional needs. Liquid media are used when there is a need for a large volume of cells or for growth measurements (230).

Most of the bacteria are harmless and inhabit the human body without causing adverse effects. The great majority are found in skin and gut and most of them are beneficial. Nevertheless, there are also a large number of infectious bacteria capable of causing morbidity and mortality, especially in countries with ineffective health care conditions and in immunocompromised patients. Others, which seem less harmful at first sight, are largely responsible for nosocomial infections. Among these are, for example, some serotypes of *Escherichia coli*, a Gram$^-$ bacteria responsible for gastroenteritis and urinary tract infections, and *Enterococcus faecalis*, a Gram$^+$ one with high resistance to antibiotics, responsible for endocarditis, meningitis and urinary tract infections (229, 231).

2.4.5.2. Antibacterial activity

There are a number of ways for bacteria to cause disease. In addition to superficial infections, bacteria can invade sterile locations, such as the bladder or blood, and through them invade vital organs as brain and heart. Despite the specific (immune system) and non-specific defenses of human body against pathogens, most of the times the organism cannot overcome an infection by itself and the inflammatory reaction normally arising from its defense process leads to fibroses and organs damage (232). In these situations, there is a need for the use of antibacterial agents (generally referred to as antibiotics), which are small molecules that interfere with normal life process of bacteria and act selectively towards human cells. The first antibiotic compound, penicillin, was discovered in 1928 by the physician Alexander Fleming, in a culture of a fungus from the genus *Penicillium* (233). Although the first antibiotics were from natural sources, most of the ones used today are chemical and semi-synthetic modifications of the original natural molecules. Some important aspects related to antibiotics effectiveness have to be considered, namely, their ability to directly kill the infectious organism (bactericidal or lethal compounds) or to slow down its growth (bacteriostatic compounds), in such a way that they will not represent danger to the host organism, since the immune system is effective in reducing the number of microorganisms in an infection, when it is residual (234).

The emergence of resistance of bacteria to antibacterial drugs, and the rising side effects and allergic reactions to the compounds commonly used in therapeutics, lead the scientific community to the search for new compounds with antibacterial properties.

Besides this, and considering the natural products potential, natural antibiotics or antibacterial substances are preferred over synthetic ones. In the past few years, marine macroalgae have been looked as a source of antibacterial compounds (235). The antibacterial activity of compounds isolated from macroalgae (236-242) and of macroalgae extracts (243, 244) has been screened. Although in this sense more attention has been paid to red algae, scientists' interest is being redirected to brown algae and their polyphenols. Some existing studies prove that these compounds are effective against a wide range of pathogenic bacteria (106, 245). Sandsdalen and co-workers isolated a polyhydroxylated fucophlorethol from a species of *Fucus*, which presented bactericidal activity against $Gram^-$ and $Gram^+$ bacteria without causing hemolytic effect (246). Other phlorotannins, mainly isolated from macroalgae belonging to the genus *Ecklonia*, have been proved as antibacterials: eckol, dieckol, 8, 8'-bieckol, phlorofucofuroeckol A, fucofuroeckol A, dioxinodehydroeckol and 7-phloroeckol (106, 108, 247-249). Although the mechanism by which phlorotannins exert antibacterial activity has not yet been defined, it is believed that, as verified for terrestrial tannins, these compounds can act by forming complexes with bacterial proteins and carbohydrates and inhibit extracellular microbial enzymes (250, 251).

2.4.5.3. Fungi

Fungi kingdom is composed by a wide variety of eukaryotic organisms with a diverse range of forms and functions. One of the main reasons for these organisms being grouped in a different kingdom is related to the presence of chitin in their cell walls (252). Based on their morphologic, physiologic and reproductive structures characteristics, fungi are classified into seven phyla: Microsporidia, Chytridiomycota, Blastocladiomycota, Neocallimastigomycota, Glomeromycota, Basidiomycota and Ascomycota (253, 254). Of these, Ascomycota have been isolated from the most extreme environments of earth and constitutes the largest, most diverse and ubiquitous phylum, occurring in a wide variety of ecosystems. Species belonging to this phylum are characterized by the presence of a specialized sac-like structure (ascus) in which meiotic spores (ascospores) are produced. Nevertheless, a large number of Ascomycota occur as a single celled yeast that reproduce by budding or binary fission (**Figure 17**) (255).

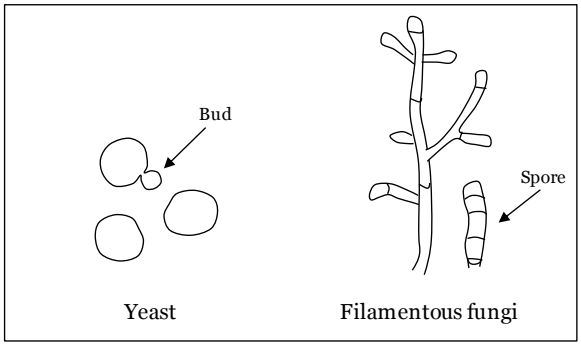

Figure 17. General representation of the two morphologies of Ascomycota.

Among the great variety of species belonging to this phylum, dermatophytes (filamentous fungi) and yeasts have an especial importance from the health care point of view, since they are common causes of skin and mucosa diseases in animals and humans (256).

Dermatophytes

Dermatophytes are Ascomycota belonging to the family Arthrodermataceae (250). Many of these organisms are saprophytic soil fungi, without any pathogenic feature for humans and animals (257). Typically anthropophilic fungi do not invade living tissues and are known to cause superficial infections (dermatophytosis) on the keratinized *stratum corneum* of skin, hair and nails. They have the capacity to obtain nutrients from keratinized tissues due to the presence of keratinases, which are responsible for hydrolyzing keratin (258). Attending to their slow growth, infections caused by dermatophytes are normally lasting and difficult to treat, being particularly demanding on immunocompromised patients.

The physical and chemical structure of the skin constitutes the first defense mechanism against dermatophytes. Nevertheless, when this barrier is compromised, an exacerbation of the infection can occur and the fungi can become invasive. Inflammation occurs together with the fungal infection, which becomes enhanced and more difficult to treat. A severe widespread dermatophytosis, besides being difficult to control, can present unusual morphologies and multiple lesions (259). As filamentous fungi, dermatophytes form organized hyphae, composed by glucans and chitin. Hyphae are cylindrical structures, septated or not, that grow at the apices. New hyphae occur by branching or bifurcation, leading to the development of a mycelium. They reproduce by sexual and

asexual means and produce spores, which morphology and pigmentation can be used for species identification (**Figure 17**) (260).

There are three genera of pathogenic anthropophilic dermatophytes: *Trichophyton*, *Microsporum* and *Epidermophyton*. *Trichophyton* are related to several dermatophytosis, namely tinea (*Tinea pedis*, *Tinea capitis* and *Tinea barbae*), ringworm and onychomycosis. Of these, *Trichophyton rubrum* is the most common dermatophyte affecting fingers and nails. *Trichophyton mentagrophytes*, which can be seen as a zoophilic form of *T. rubrum*, has a wide range of animal hosts and is responsible for the production of scalp lesions in humans. *Microsporum* species are also responsible for causing tinea and ringworm. Pets are a recognized reservoir of some *Microsporum* species, such as *Microsporum canis* and *Microsporum gypseum*, responsible for causing ringworm on dogs and cats. *Epidermophyton* is represented by only one species, *Epidermophyton floccosum*, which is known to cause cutaneous mycosis like tinea (257, 259).

Yeasts

More than 1500 yeast species have been identified, which represents only a fraction of yeast biodiversity on Earth (261). Yeasts are members of the phylum Ascomycota and, even not as widespread as bacteria, these organisms can be found in plants, animals, soil, water and atmosphere, and also in extreme environments (e.g. osmophilic and halotolerant yeasts). As non-motile organisms, yeasts rely on aerosols, animal vectors and human activity for their natural scatter (262).

Morphologically different from dermatophytes, yeast cells present an ellipsoidal shape, which compartmentalization typifies that of eukaryotic cells, with nucleus, mitochondria, Golgi apparatus, vacuoles and peroxisomes. The structural organization of the intracellular components is maintained by a cytoskeleton. The cellular contents are encased by an envelope comprising plasma membrane, periplasm and cell wall (**Figure 18**) (262, 263).

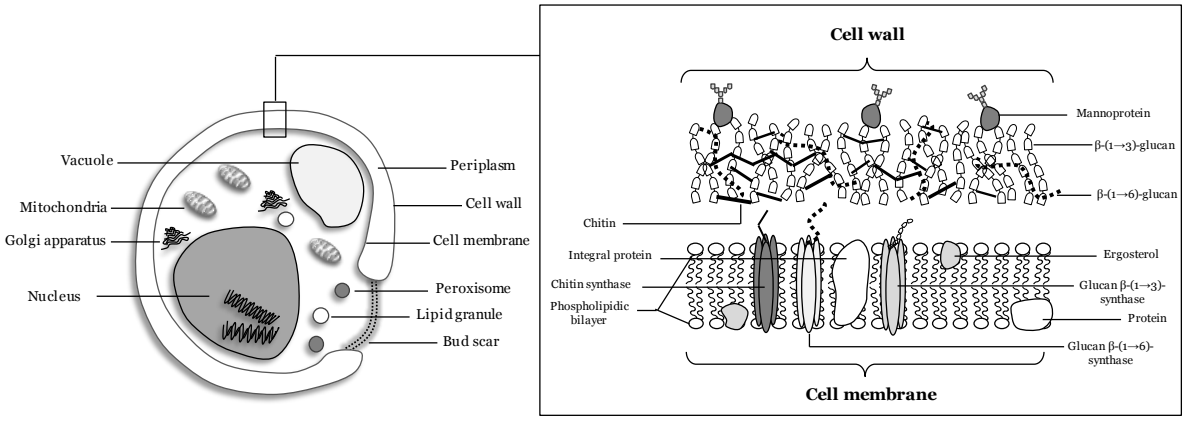

Figure 18. Schematic representation of a yeast cell. Detail of the cell membrane and cell wall composition.

The cell wall comprises about 1/4 to 1/5 of total yeast dry mass. It is mainly composed by polysaccharides (mainly β-(1→3) and β-(1→6)-glucans), β-(1→3)-glucans being in higher amount. The cell wall has minor amounts of chitin, which can be found in major concentration in the bud scar (**Figure 18**). The amounts of chitin in yeasts are lower than those of filamentous fungi and some yeasts completely lack chitin. Anyway, the amounts of the chemical constituents of yeast cell walls vary according to the strain, cell age and growth conditions (262). Concerning the yeast cell membrane, in a general way, it is composed by a phospholipidic bilayer with proteins, enzymes and sterols (**Figure 18**). Ergosterol is a bioregulator of membrane fluidity and asymmetry and, consequently, of membrane integrity; cells are unable to grow in its absence (256). Due to their location, characteristics and function, the cell membrane and cell wall components are the main targets for the action of antifungal drugs (264).

Yeasts from the genus *Candida*, particularly *Candida albicans*, are the most important agents causing yeast infection. This opportunistic pathogen is normally associated with mucosal, cutaneous and nail infections (normally known as candidiasis), but can cause acute or chronic invasive infections in immunocompromised or debilitated individuals. This yeast is part of human flora and, in most cases, infections are derived from the individuals own reservoir in mouth, gastrointestinal tract, lower genital tract or skin. Despite the mechanisms that can prevent the organism from establishing infection, (physical, chemical and immunological), in some situations yeasts can establish infection. When there are serious impairments of host defenses, life-threatening invasive infections can occur (256). The ability of *Candida* species to cause disease depends upon two main factors: survival and virulence. Virulence factors allow the organisms to invade new

tissues, evading phagocytic cells and causing symptoms of infection. One of the most important virulence factors in *Candida* species is the alteration of cell morphology, i.e. dimorphism. Within the genus *Candida*, only the species *C. albicans* and *Candida dubliniensis* are able to undergo dimorphic transition. Dimorphism is the ability to produce either yeast cells (blastospores) or filamentous forms (hyphae or germ tubes and pseudohyphae) (265) (**Figure 19**). The production of germ tubes by *C. albicans* is involved in the microorganism pathogenesis, as these are responsible for the adhesion of yeast cells to the mucosa, turning *Candida* infections more difficult to overcome. In most cases, the inhibition of the production of the germ tube is sufficient to treat disseminated candidiasis (266).

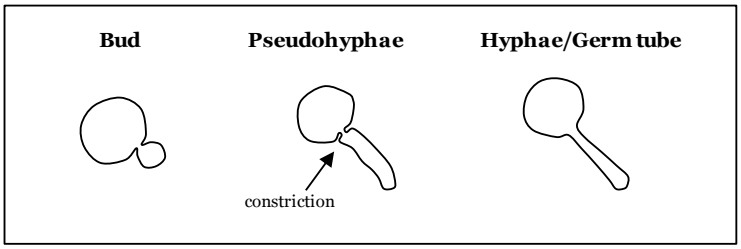

Figure 19. Morphology of a bud, pseudohyphae and hyphae/germ tube in dimorphic yeasts.

Germ tubes are considered positive when they are at least as long as the diameter of the blastospore (mother cell), being negative when showing a constriction at the point of connection to the mother cell, typical of an incomplete budding and known as pseudohyphae (265, 267).

2.4.5.4. Mechanism of antifungals' action

The study of antifungals' mechanism of action constitutes an important strategy for limiting the emergence of resistance to the commercially available agents, as well as to develop safer and more potent compounds in the future. Understanding the mechanism of action of antimicrobial agents is an important tool to understand the mechanism of microorganisms' resistance. As referred above, the fungal membrane and cell wall components are the most important targets for the action of antifungal drugs (156).

Compounds active on fungal cell membrane

Concerning the fungal membrane, there are three main groups of antifungals acting in this target: azoles, polyenes and allylamines. All of them owe their antifungal action to

the interaction with ergosterol. This interaction can result in the depletion of ergosterol in the fungal membrane by affecting sterols biosynthesis, *via* inhibition of the enzyme 14α-demethylase. The antifungals interaction can also occur in the early stages of ergosterol biosynthesis, leading to the accumulation of squalene. Compounds that act by this mechanism lead not only to the depletion of ergosterol, but also to the accumulation of sterol precursors. A direct action on the membrane ergosterol molecule can also occur, and some antifungals can form aqueous pores that cause alteration of cell permeability, leakage of vital cytoplasmic components and death (156).

Several mechanisms are related to fungal resistance to antifungals that owe their action to the interaction with ergosterol, namely modifications or over-expression of target enzymes, production of modified sterols and cross-resistance (156).

Compounds active on fungal cell wall

Fungal cell wall contains compounds that are unique in fungal kingdom and constitute a good target for antifungal drugs. Two main groups of antifungals act on the cell wall: the ones inhibiting glucans and the ones inhibiting chitin synthesis.

Antifungals responsible for the inhibition of glucans synthesis include specific inhibitors of β-(1→3)-glucans synthase. These compounds are fungicidal lipopeptides that promote cytological changes in fungi and lead them to grow as pseudohyphae, thickened cell wall and buds attached to mother cells (264). Chitin inhibitors include compounds structurally similar to *N*-acetylglucosamine (chitin basic units linked by β-(1→4) linkages) that act as competitive inhibitors of chitin synthases in pathogenic fungi and insects. Because of their non-toxic and easily degradable properties, chitin inhibitors are potent antifungal agents and insecticides in agriculture and human therapy (268).

A drug can also owe its antimycotic effect to the interaction with the respiratory chain processes in mitochondria. These organelles are responsible for the generation and regulation of ROS, Ca^{2+} homeostasis, regulation of apoptosis and metabolic processes, being also responsible for more than 90% of cellular ATP production (269). Compounds with the capacity to affect mitochondrial respiratory chain can be seen as potential cell growth inhibitors and capable of trigger cell death (269-271). There is currently no antifungal drug owing its primary mechanism of action to the interference with mitochondria activity. Nevertheless, besides the primary mechanism of action, some antifungal drugs like azoles and polyenes can act in more than one target, having some effect on mitochondria activity (272).

2.4.5.5. Antifungal activity

The antifungal activity of seaweeds extracts and isolated compounds has not been extensively studied, mainly because in the past few years more attention has been paid to pathogenic bacteria, which is, by far, more explored (155). Nevertheless, there are some studies concerning the antifungal activity of brown seaweeds extracts and few studies concerning phlorotannins. Guedes and co-workers evaluated the antifungal activity of different seaweeds crude extracts against dermatophytes and *Candida* sp., concluding that the dichloromethanolic, MeOH and ethanol extracts were the most active and, in a general way, dermatophyte were more sensitive than *Candida* sp. (273). Brown seaweeds from the Gulf of Mannar and from the Aegean Sea were also studied yielding roughly the same results (102, 109). Several other studies concerning the screening of antifungal activity of brown seaweeds extracts were developed (104, 105, 274-276). The antifungal activity of some brown seaweeds extracts has been attributed to phlorotannins (277, 278). Lee and co-workers isolated dieckol from a species of *Ecklonia* and tested it against *T. rubrum*. The compounds presented fungicidal activity and that effect was attributed to the loss of dermatophyte membrane integrity (249). To date, there are no studies concerning the mechanism of action of extracts or isolated phlorotannins. Despite the study of the mechanism of action of extracts may seem abstract due to the possibility of acting in multiple cell targets, it can provide an added value to therapeutic. Acting in different cell targets reduces the microorganisms' resistance and can lead to better therapeutic responses.

3. AIMS OF THE DISSERTATION

1. Characterization of seaweeds species in terms of sterols:

1.1. Qualitative and quantitative sterols profile.

1.2. Establishment of a relationship between the sterols profile and the nutritional value of the studied species.

2. Characterization of brown seaweeds in terms of phlorotannins:

2.1. Quantitative phlorotannins profile determination by the DMBA assay.

2.2. Screening of the anti-inflammatory and NO scavenging capacity of purified phlorotannins extracts, *in vitro,* in cell and cell-free systems.

2.3. Screening of the antimicrobial activity of purified phlorotannins extracts.

3. Qualitative analysis of phlorotannins by HPLC-DAD-ESI/MS^n:

3.1 Establishment of the phlorotannins profile of the most promising bioactive species by HPLC-DAD-ESI/MS^n.

3.2. Determination of the antioxidant activity of purified phlorotannins extracts and their Haase inhibitory capacity.

4. Approach to the mechanism of antifungal action of purified phlorotannins extracts:

4.1. Determination of the antifungal action of purified phlorotannins extracts against a wide range of dermatohytes and yeasts.

4.2. Approach to the mechanism of antifungal action of phlorotannins extracts by the evaluation of their effect on the cell wall, membrane composition and metabolic network of microorganisms.

5. Compounds isolation of the most promising seaweed species:

5.1. Isolation of compounds by chromatographic methods and structure elucidation.

5.2. Evaluation of the anti-inflammatory activity of the isolated compounds.

PART II

EXPERIMENTAL SECTION

4. EXPERIMENTAL SECTION

4.1. Standards and reagents

Cholesterol (99%), desmosterol (87%), ergosterol (75%), fucosterol (95%), stigmasterol (95%), β-sitosterol (98%), campesterol (65%), phloroglucinol (98%), sodium pyruvate, thiazolyl blue tetrazolium bromide (MTT), β-nicotinamide adenine dinucleotide reduced form (NADH), sodium nitroprusside dehydrate (SNP), sulfanilamide, toluene, MeOH, dimethyl sulfoxide (DMSO), 2,4-dimethoxybenzaldehyde (DMBA), *p*-anisaldehyde, dichloromethane (DCM), ETOAc, LPS from *Salmonella enteric*, trypan blue solution (0.4%), sodium chloride (NaCl), 3-(N-morpholino) propanesulfonic acid (MOPS), N-acetylglucosamine, D-(+)-glucosamine hydrochloride, rhodamine 123 (RHO), 3-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate, ammonium sulfamate (NH4 sulfamate), ferric chloride ($FeCl_3$), formic acid, HAase from bovine testes (type IV-S), HA sodium salt from *Streptococcus equi*, nitrotetrazolium blue chloride (NBT), phenazine methosulfate (PMS), L-ascorbic acid, sodium formate, trizma hydrochloride (Tris-HCl), bovine serum albumin (BSA), dexamethasone and curdlan from *Alcaligenes faecalis* were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cellulose microcrystalline for thin-layer chromatography, silica gel for thin-layer chromatography 60 G (particle size 5–40 mm) and 60M F_{254}, N-(1-naphthyl) ethylenediamine, acetone, n-hexane, acetic acid (glacial), HPLC-grade MeOH, acetonitrile, potassium di-hydrogen phosphate buffer, di-sodium tetraborate, iron (II) sulfate ($FeSO_4 \cdot 7H_2O$), sodium nitrite ($NaNO_2$), potassium hydrogen sulfate ($KHSO_4$), n-butanol (BuOH) and 4-dimethylaminobenzaldehyde (DMAB) were obtained from Merck (Darmstadt, Germany).

Hydrochloric acid (HCl), *ortho*-phosphoric acid (H_3PO_4), anhydrous sodium sulfate, ethanol, diethyl ether, KOH and sodium hydroxide (NaOH) were purchased from Panreac (Castellar del Valle`s, Spain).

Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's phosphate buffered saline (DPBS), heat inactivated fetal bovine serum (FBS) and Pen Strep solution (Penicillin 5000 units/mL and Streptomycin 5000 mg/mL) were from Gibco (Invitrogen, Paisley, UK).

Mueller Hinton broth (MHB) and Mueller Hinton agar (MHA) media were purchased from Liofilchem (Teramo, Italy). Sabouraud dextrose agar (SDA) was from Bio-Mèrieux (Marcy L'Etoile, France). RPMI-1640 broth medium (with L-glutamine, without bicarbonate, and with the pH indicator phenol red) was purchased from Biochrom AG

(Berlin, Germany). Gentamicin was purchased from Appli- chem (Darmstadt, Germany) and fluconazole was kindly provided by Pfizer (Barcelona, Spain). Proline and aniline blue water soluble ($C_{37}H_{27}N_3Na_2O_9S_3$) were from Fluka (Buchs, Sankt Gallen, CH). Yeast nitrogen base was from Difco (New Jersey, USA). LA was from Calbiochem (San Diego, USA).

The murine macrophage-like cell line RAW 264.7 was from the American Type Culture Collection (LGC Standards S.L.U., Spain).

H_2O was deionized using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

4.2. Seaweeds material

Individuals of eighteen seaweeds species (ten Phaeophyta, three Chlorophyta and five Rhodophyta) (**Figures 20** and **21**) were randomly collected in the coast of Peniche (west Portugal) and identified by specialists from the School of Tourism and Maritime Technology of the Polytechnic Institute of Leiria, Portugal. Each seaweed sample corresponded to a mixture of 3–4 individuals in the same stage of development. With the exceptions of *Cystoseira usneoides* (Linnaeus) M. Roberts and *Cystoseira nodicaulis* (Withering) M. Roberts, collected in 2009, all samples were collected in 2008. *Sacchorhyza polyschides* (Lightfoot) Batters was collected in June, *C. usneoides* and *C. nodicaulis* in November and the remaining species in September. After collection, samples were placed on ice and immediately transported to the laboratory in insulated sealed ice-boxes, to protect them from heat, air and light exposition. The fresh biomass was cleaned, in order to remove epiphytes and encrusting material, washed with NaCl aqueous solution (3.5% m/m) and kept at -20 ºC, prior to their lyophilization in a Labconco 4.5 Freezone apparatus (Kansas City, MO, USA). The dried material was powdered (<910 μm) and kept in the dark, in a desiccator, until preparation of the extracts for analysis.

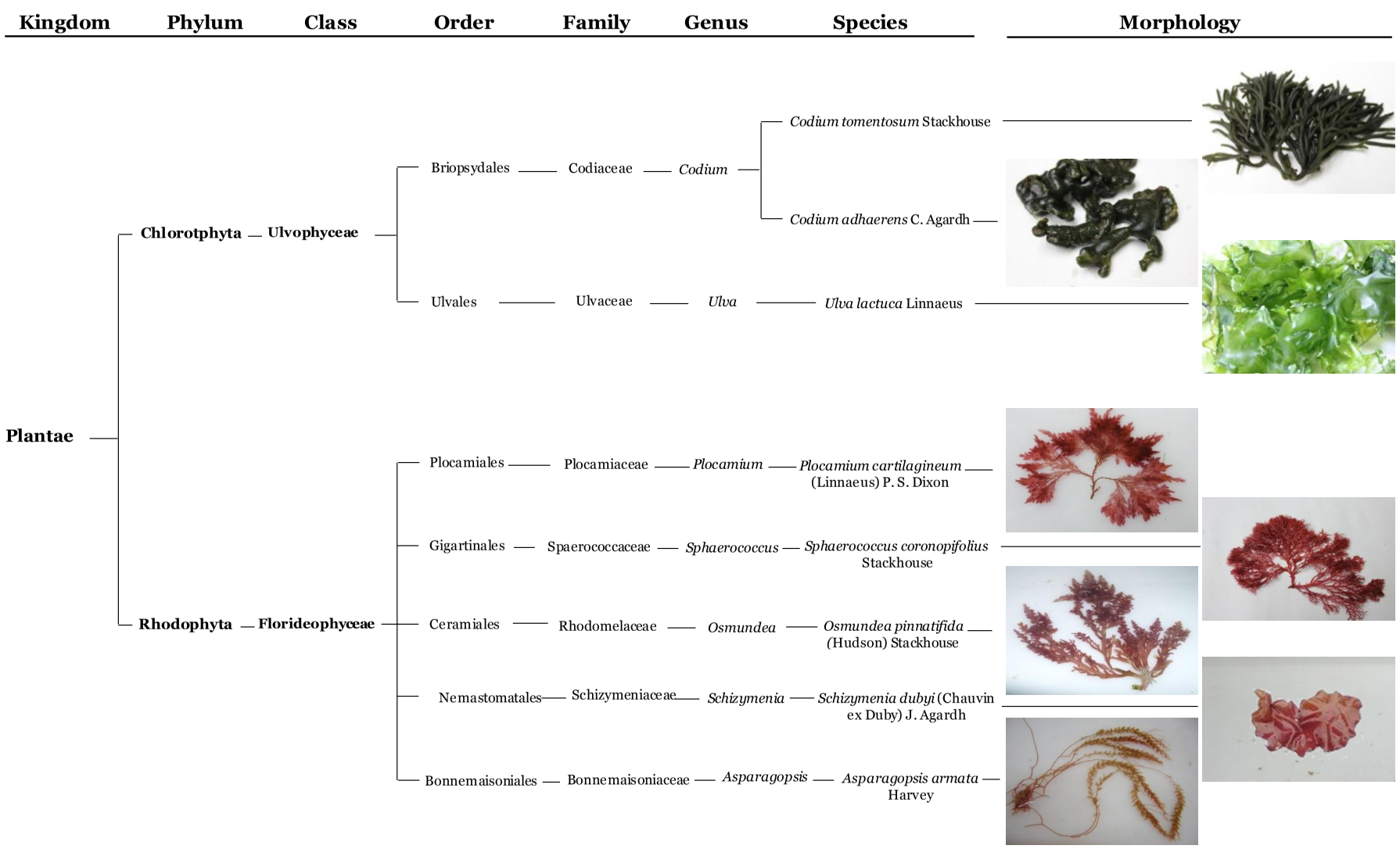

Figure 20. Phylogenetic representation of the Chlorophyta and Rhodophyta samples collected in the Portuguese west coast. *(Photographs of Graciliana Lopes).*

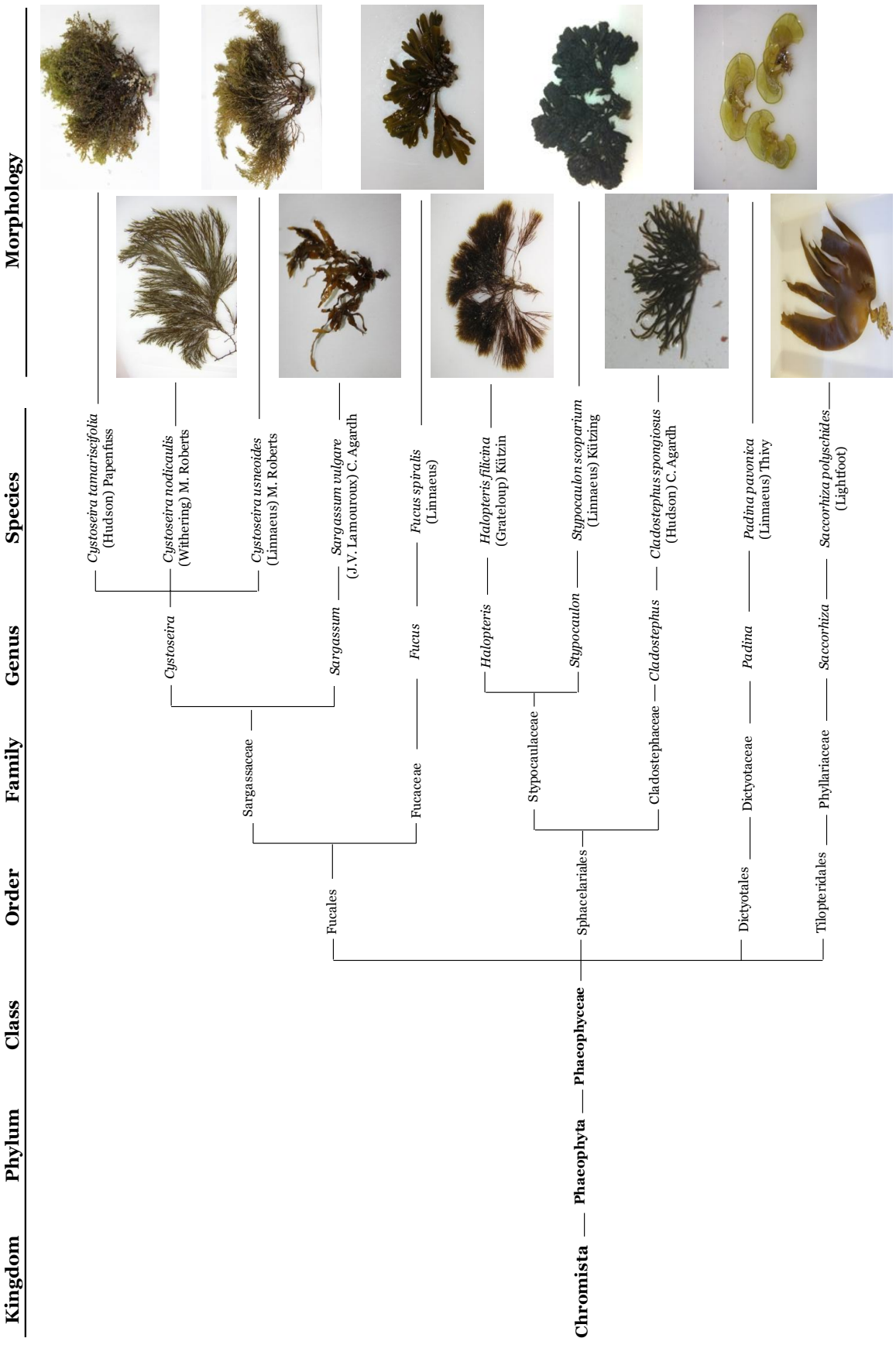

Figure 21. Phylogenetic representation of the Phaeophyta samples collected in the Portuguese west coast. *(Photographs of Graciliana Lopes).*

4.3. Sterols profile

4.3.1. Extraction

All the seaweeds samples (**Figures 20** and **21**) were analyzed for their qualitative and quantitative sterols profile. In order to maximize the extraction of sterols the algae material was subjected to an extraction process that allowed the simultaneous saponification of sterols and their consequent conversion to the free form.

The saponification process consists in the reaction of esterified sterols with a strong base, in order to break the ether linkages between the sterol moiety and the fatty acid. The most suitable conditions for extraction and saponification (or alkaline hydrolysis) were established by testing several combinations of KOH and n-hexane. For this, *O. pinnatifida* (the sample available in highest amounts) was processed following a described procedure (79). First, the powdered sample was extracted and saponified with varying volumes of ethanolic KOH 10% (10, 20, 30, 40, and 50 mL). After this first step, the hydrolysate was extracted with n-hexane, varying the number of extractions (1, 2, 3, and 4 times with 10 mL). Best results were obtained using 40 mL of ethanolic KOH 10% for saponification and 3 x 10 mL of n-hexane for the extractive processes. Optimal conditions were achieved on the basis of peak areas obtained in HPLC–DAD chromatograms.

After optimizing the extraction/saponification conditions, 0.5 g of powdered lyophilized material of each seaweed species were weighed out in an Erlenmeyer flask and saponified with 40 mL of ethanolic KOH (10%) for 1 h, under constant shaking (1000 rpm) and temperature (70 ºC). The Erlenmeyer was left to cool down to room temperature and the mixture was centrifuged (Labofuge Ae, Heraeus Sepatech GmbH, Osterode, Germany) during 3 min at 5000 rpm and decanted. The mixture was extracted with 3 x 10 mL of n-hexane, in a separation funnel, in order to remove the free sterols. The organic phase containing the sterols was dehydrated with anhydrous sodium sulfate, evaporated to dryness under reduced pressure (at 30) and kept in the freezer until analysis by HPLC-DAD. Immediately before chromatographic analysis, the residue was dissolved in 1 mL of 30:70 (v / v) MeOH:acetonitrile mixture and filtered through a 0.22 μm membrane (Millipore).

4.3.2. Analysis by HPLC-DAD

The HPLC–DAD system comprised a liquid chromatographer equipped with models 302 and 305 pumps (Gilson Medical Electronics, Villiers le Bel, France), a 20 μL loop and a DAD (170, Agilent, Villiers le Bel, France) controlled by Unipoint system Software (Gilson Medical Electronics). Separation was performed using a reversed-phase Hypersil ODS (20 x 0.4 cm, 5 μm particle diameter; Teknokroma, Barcelona, Spain) column. The elution conditions used for sample analysis were selected on the basis of preliminary trials to establish the most suitable ones. The temperature and flow rate were optimized in order to achieve the best separation of sterols. Room temperature and temperature set at 30, 35, and 60 ºC with a column heater (Jones-Chromatography model 7981, Mid Glamorgan, UK), as well as flow rates of 0.8, 1.0 and 1.2 mL/min were assayed. The best separation was achieved at room temperature, with a 0.8 mL/min flow.

Several gradients using MeOH and acetonitrile were also tested. It was not possible to obtain a stable base line at the wavelength of detection (205 nm). For this reason, isocratic elution with MeOH:acetonitrile (30:70) was performed. Spectral data from all peaks were accumulated in the 190–400 nm range. Peak purity was checked by the software contrast facilities. Sterols were identified by comparing their retention times and UV absorption spectra with standards commercially obtained. Quantification was achieved by the absorbance (Abs) recorded in the chromatograms at 205 nm, except for ergosterol, which was determined at 280 nm.

4.3.3. Validation of the methodology

In order to validate the optimized methodology, calibration curves were performed with four different concentrations of standard solutions, selected as representative of the range of compounds' concentrations in the samples: 125–1000 μg/mL for fucosterol, 71–567 μg/mL for cholesterol, 20–128 μg/mL for desmosterol, 143–572 μg/mL for ergosterol, 65–522 μg/mL for β-sitosterol, 125–500 μg/mL for campesterol and 125–1000 μg/mL for stigmasterol. The calibration plots showed a good correlation: r^2 values for all sterols were higher than 0.99, with the exceptions of ergosterol and β-sitosterol (0.9839 and 0.9884, respectively). The limit of detection (LOD) ($3S_o/b$) and the limit of quantification (LOQ) ($10S_o/b$, where S_o is the standard deviation of signal-to-noise ratio and b is the slope of the calibration plot) for the analyzed sterols are shown in **Table 1**.

Table 1. Limit of detection (LOD)[a], limit of quantification (LOQ)[a], regression equations and r^2 for the different sterols.

Sterol	LOD	LOQ	Regression equations	r²
Desmosterol	5.94	19.82	$y = 353176374.3\,x + 2799435.6$	0.9987
Ergosterol	1.39	4.63	$y = 365654626.4\,x + 34387743.3$	0.9839
Fucosterol	4.03	13.44	$y = 87253639.0\,x + 1500128.4$	0.9997
Cholesterol	4.43	14.78	$y = 167654599.1\,x + 8709832.1$	0.9945
Campesterol	3.94	13.12	$y = 64900831.6\,x - 738494.7$	0.9960
Stigmasterol	27.95	93.16	$y = 252456154.9\,x + 17313724.1$	0.9946
β-Sitosterol	3.39	11.28	$y = 103086932.3\,x + 5049720.1$	0.9884

[a] μg/mL.

In order to evaluate sterols recovery, cholesterol was used, as it was the standard available in highest amounts and with the highest purity degree. Cholesterol was also found in intermediate amounts in all samples and was not co-eluting with other compounds. For this purpose, known amounts of cholesterol (0.1, 0.2 and 1 mg) were added to a sample, which was then treated under the optimized conditions. The procedure was repeated five times and the total recovery of cholesterol corresponded to 78, 91, and 93%, respectively. Repeatability was checked by analyzing five times the same sample, by the same analyst, within the same day. The relative standard deviation (RSD) was lower than 8% (**Table 2**), indicating a good repeatability of the procedure.

Table 2. Evaluation of the method precision (RSD)[a].

Sterol	Intra-day	Inter-day
Desmosterol	0.5	7.9
Ergosterol	7.9	11.6
Fucosterol	1.7	3.4
Cholesterol	4.5	7.1
Campesterol+ Stigmasterol	6.2	13.7
β-sitosterol	0.7	15.2

[a]%

Intermediate precision on five different days (five injections a day) was also found to be satisfactory, since RSD varied between 3 and 15% (**Table 2**). Method selectivity was assessed by DAD (which ensured peak purity) and by authentic standards chromatograms.

Interferents were ruled out by verifying the absence of peaks in the retention time of the studied analytes. The optimization of the methodology ensured obtaining a rich sterol fraction, as revealed by the chromatograms, in which only peaks corresponding to compounds with UV spectra characteristic of sterols were noticed. The analysis of the same sample in different periods revealed no alteration of the chromatograms.

4.4. Phlorotannins profile

Once phlorotannins are exclusive of brown seaweeds, all the ten brown seaweeds species, belonging to the orders Fucales, Sphacelariales, Dictyotales and Tilopteridales (**Figure 21**) were used for phlorotannins quantification and screening of the anti-inflammatory and anti-microbial activity. Of them, only the most promising species were selected for characterization by LC-MS and for further biological activities evaluation.

4.4.1. Extraction

The conditions used for phlorotannins extraction were adapted from those proposed by Koivikko and co-workers (118). In order to maximize the efficiency of extraction, lipids were removed from aliquots of 0.5 g of powdered lyophilized material of each sample. For this purpose, 1 mL of n-hexane was added to the powdered sample, in a falcon tube, and hand shacked for about 1 min. The mixture was centrifuged (5 min at 4000 rpm) in a Rotofix 32 A Hettich centrifuge and the supernatant removed. The n-hexane treatment was repeated three times.

After n-hexane removal, the defatted material was extracted four times with 10 mL of a solvent mixture consisting of acetone:H_2O (7:3), for 1 h at 400 rpm, followed by centrifugation for 5 min at 4000 rpm. The organic fractions were combined into an Erlenmeyer and evaporated to dryness under reduced pressure, at 30 ºC.

4.4.2. Purification

The conditions used for extracts purification were adapted from those proposed by Fairhead and colleagues (121). The dried acetone:H_2O extract was resuspended in 30 mL of MeOH and adsorbed into cellulose (approximately 2 times the residue's weight) by gentle shaking. The suspension was dried under reduced pressure, at 30 ºC, until complete cellulose powder detachment. After cooled to room temperature, the mixture was washed with aliquots of 25 mL of toluene to remove pigments, until the filtrate run clear. Then, cellulose was rinsed with 30 mL of acetone:H_2O (7:3) to release the adhered

phlorotannins. After centrifugation (5 min at 4000 rpm), the supernatant was evaporated to dryness under reduced pressure, at 30 ºC, yielding the purified phlorotannins extract.

4.4.3. Quantification by DMBA assay

The methodology used for phlorotannins quantification was adapted from that proposed by Stern and co-workers (124). The working reagent was prepared by mixing equal volumes of DMBA (2%, m/v) and HCl (6%, v/v) prepared in glacial acetic acid. Purified phlorotannins extracts were dissolved in H_2O with 10% DMSO. Aliquots of each extract (50 µL) were mixed with 250 µL of working reagent in a 96-wells plate. The reaction was conducted at room temperature, in the dark, during 60 min. After the incubation period, the Abs was determined at 515 nm in a Multiskan Ascent plate reader (Thermo Electron Corporation).

The quantitative determination of phlorotannins was carried out using phloroglucinol as external standard. A series of seven phloroglucinol concentrations, ranging between 0.98 and 62.50 µg/mL, was used to obtain a linear calibration curve ($y = 0.0254x$) presenting an r^2 value of 0.9998. In order to evaluate phlorotannins recovery, phloroglucinol was used, as it was the only standard commercially available. Known amounts of phloroglucinol (0.250 and 0.500 mg) were added to the powdered lyophilized macroalgae material and treated under the conditions described for extraction and purification. The procedure was repeated 3 times and the total recovery of phloroglucinol ranged between 88 and 95%.

4.4.4. Characterization by LC-MS

Of the ten brown seaweeds samples, *C. nodicaulis*, *C. tamariscifolia*, *C. usneoides* and *F. spiralis* were selected for the characterization of their phlorotannins profile by LC-MS. Chromatographic analysis were carried out on a Luna C18 column (250 × 4.6 mm, 5 µm particle size; Phenomenex, Macclesfield, UK). The mobile phase consisted of two solvents: 1% formic acid in H_2O (A) and acetonitrile (B). Elution was performed under the following gradient: 0–10 min, 0% B; 30 min, 30% B; 35 min, 80% B; 40 min, 80% B; 42 min, 0% B; 52 min, 0% B. The flow rate was 1 mL/min and the injection volume was 20 µL. Spectral data from all peaks were accumulated in the range 240–400 nm and chromatograms were recorded at 280 nm. The HPLC-DAD-ESI/MS^n (n = 1–2) analysis were carried out in an Agilent HPLC 1100 series equipped with a diode array detector and mass detector in series (Agilent Technologies, Waldbronn, Germany). The HPLC consisted of a binary pump (model G1312A), an auto-sampler (model G1313A), a degasser (model G1322A) and a photodiode array detector (model G1315B). The HPLC system was

controlled by the ChemStation software (Agilent, v. 08.03). The mass detector was an ion trap spectrometer (model G2445A) equipped with an electrospray ionization interface and was controlled by LCMSD software (Agilent, v. 4.1). The ionization conditions were adjusted at 350 °C and 4 kV for capillary temperature and voltage, respectively. The nebulizer pressure and flow rate of nitrogen were 65.0 psi and 11 L/min, respectively. The full scan mass covered the range from *m/z* 100 up to *m/z* 1500. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 up to 2 V. MS data were acquired in the positive ionization mode.

4.5. Biological activities of purified phlorotannins extracts

4.5.1. Anti-inflammatory activity

The anti-inflammatory capacity and the toxicity of purified phlorotannins extracts was evaluated in a macrophage cell line (RAW 264.7). The amount of NO in cellular culture medium and in a cell-free system was measured. The metabolic activity of macrophages was evaluated by the MTT assay and viability was checked by measuring the extracellular lactate dehydrogenase (LDH).

Purified phlorotannins extracts (dissolved in H_2O with 10% DMSO) were sterilized by filtration through a 0.22 µm membrane before cells treatments. The serial dilutions of the extracts were prepared with DMEM culture medium supplemented with 10% FBS and 2% Pen Strep solution, under aseptic conditions.

4.5.1.1. Cell culture and treatments

Murine macrophage RAW 264.7 cells were cultured in 75 mL cell flasks at 37 °C, in DMEM culture medium supplemented with 10% FBS and 2% Pen Strep solution, in a humidified atmosphere of 5% CO_2. After confluence, cells were detached from the flask with a countersink and inoculated at a density of 150 000 cells/well into 48-wells plates. Cells were cultured until confluence under the same conditions.

After reaching confluence (about 2 days), cells were pre-treated with 975 µL of different concentrations of purified phlorotannins extracts or vehicle for 1 h. After that, 25 µL of LPS (or vehicle) was added to each well, in order to achieve a final LPS concentration of 1 µg/mL. Cells were further incubated for 18 h at 37 °C in a humidified atmosphere of 5% CO_2 and, after this period, assayed for cell viability and NO. The final concentration of DMSO in each well was 0.5%. Four independent assays were performed in duplicate.

4.5.1.2. Cell viability

LDH assay

The LDH released into the culture medium was used as an index of cell death. After the incubation period, the culture medium was carefully removed from each well and taken to determine the activity of LDH released by death cells. Briefly, after the incubation period an aliquot of culture medium was taken to determine the activity of LDH leaked through cell membranes. The culture medium was transferred to a 96-wells plate in aliquots of 50 μL, in triplicate. 200 μL of a 150 μg/mL NADH solution was added to each well. The reaction was initiated with the addition of 25 μL of a 150 μg/mL pyruvate solution. Both LDH and pyruvate solutions were prepared in phosphate buffer (KH_2PO_4 10 mM, pH 7.4). LDH activity was determined following the oxidation of NADH during the conversion of pyruvate to lactate at 340 nm (**Figure 22**). Results are expressed as LDH activity in the medium of exposed cells, relative to control, without extract (279).

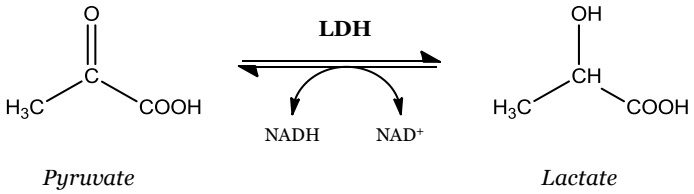

Figure 22. Reaction of interconversion of pyruvate to lactate under NADH oxidation, by the action of LDH.

MTT assay

Contrarily to LDH, which is determined in the culture medium, the cell viability measured through the MTT assay is determined in the cells. After the incubation period, RAW 264.7 cells were washed with DPBS and then incubated for 30 min with a 0.5 mg/mL MTT solution (prepared in DMEM culture medium). The yellow tetrazolium salt MTT is converted by mitochondrial dehydrogenases of metabolically active cells to an insoluble purple formazan product. This product was solubilized with DMSO and the extent of the reduction of MTT to formazan within the cells was quantified by measuring the Abs of the reaction product at 510 nm (**Figure 23**) (279).

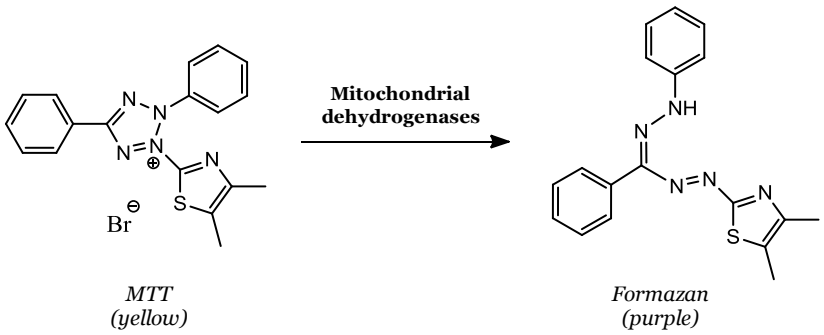

Figure 23. Reaction of conversion of the yellow MTT to the purple formazan salts, by the action of mitochondrial dehydrogenases of metabolically active cells.

4.5.1.3. NO production by RAW 264.7 cells

After the incubation period, the nitrite accumulated in the culture medium was measured as an indicator of NO production (217). Based on the Griess reaction (**Figure 24**), equal volumes (100 μL) of culture supernatant and Griess reagent [1:1 mixture (v/v) of 1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine in 2% H_3PO_4] were mixed and incubated for 10 min in the dark, at room temperature.

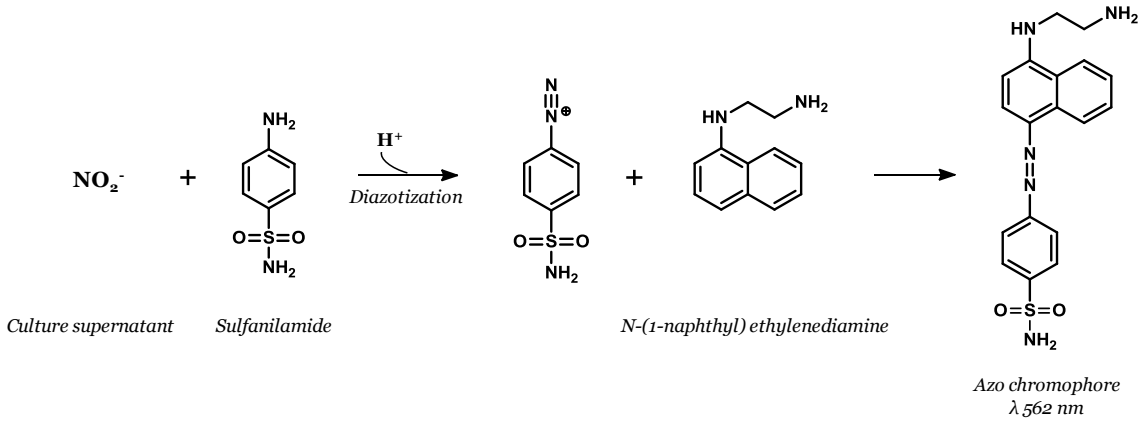

Figure 24. Measurement of NO_2^- by the Griess reaction.

The Abs of the chromophore formed during the diazotization of nitrite (NO_2^-) with sulfanilamide and subsequent coupling with naphthylethylenediamine dichloride was read at 562 nm. Control values were obtained in the absence of purified phlorotannins extracts and after addition of LPS. Four independent assays were performed in duplicate.

4.5.2. Antioxidant capacity

4.5.2.1. NO scavenging assay

The capacity of purified phlorotannins extracts to scavenge NO was also evaluated in a cell-free system. NO was generated *in vitro* from SNP and measured by the Griess reaction (**Figure 24**) (280). SNP solution (6 mg/mL) was prepared in phosphate buffer (KH_2PO_4 100 mM, pH 7.4) and mixed with the same volume (100µL) of different concentrations of purified phlorotannins extracts, in a 96-wells plate. The mixture was further incubated at room temperature for 1 h under light. After that, 100 µL of Griess reagent was added and the mixture was further incubated for 10 min in the dark. The Abs was read at 562 nm. Control values were obtained in the absence of purified phlorotannins extracts. Four independent assays were performed in duplicate.

4.5.2.2. Superoxide anion scavenging assay

Superoxide radical scavenging activity was evaluated as previously described (281). Briefly, serial dilutions of purified phlorotannins extracts were prepared; 50 µL of each dilution was transferred to a 96-wells plate and mixed with the same volume of a 166 µM NADH solution and 150 µL of NBT (43 µM). The reaction was started by the addition of 50 µL of PMS (2.7 µM). $O_2^{\bullet-}$ was generated by the NADH/PMS system. The scavenging activity of purified phlorotannins extracts was determined by monitoring their effect on the reduction of NBT induced by $O_2^{\bullet-}$ (**Figure 25**), using a plate reader working in kinetic function, at room temperature, for 2 min, at 562 nm. Controls (without extract) were included for all the assays. All reagents and samples dilutions were dissolved in phosphate buffer (19 mM, pH 7.4). Three independent assays were performed in triplicate.

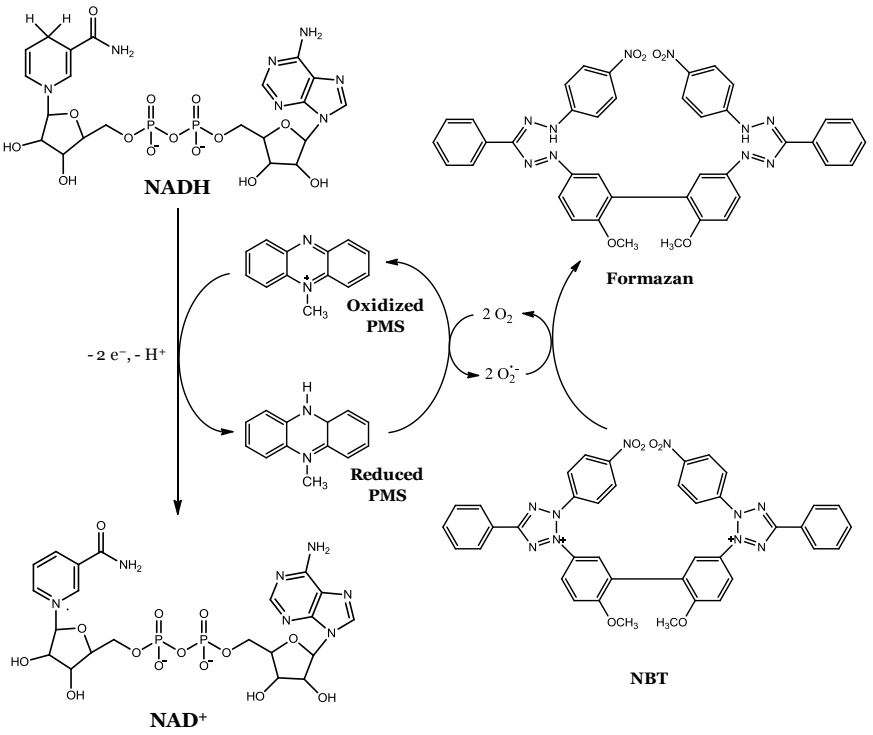

Figure 25. Schematic representation of the reduction of NBT induced by $O_2^{\bullet-}$, generated by the NADH/PMS system.

4.5.3. Phlorotannins effects on cell components and enzymes associated with the spread of inflammatory and allergic mediators and aging

The ability of purified phlorotannins extracts to act as coadjuvants in the delay of the aging process and in the spread of chemical mediators associated with several pathological states (such as inflammation and allergy) was assessed by the evaluation of their capacity to inhibit LPO and the enzyme HAase. The three *Cystoseira* species and *F. spiralis* were used in these assays.

4.5.3.1. LPO inhibition

Peroxidation of fatty acyl groups was modified from the methodologies proposed before (282, 283). The reaction was developed in test tubes. The reaction mixture contained 250 µL of LA (20 mM in ethanol), 150 µL of Tris-HCl (100 mM, pH 7.5), 50 µL of $FeSO_4.7H_2O$ (4 mM in H_2O) and 50 µL of serial dilutions of purified phlorotannins extracts prepared in distilled H_2O. LA peroxidation was initiated by the addition of 50 µL of ascorbic acid (5 mM in H_2O) and the mixture was immediately incubated for 1 h at 37 °C. After the incubation period, 1.5 mL of ethanol:ether (3:1) mixture was added to each

test tube. This mixture allows the rearrangement of the double bonds, resulting in the formation of conjugated dienes (lipid hydroperoxides). The test tubes were then vortexed and the Abs was immediately measured at 233 nm in a Helios α (Unicam) spectrophotometer, at room temperature. Positive controls (without extract) and negative controls (without LA) were performed along with the samples. Three independent assays were performed in triplicate.

4.5.3.2. HAase inhibition

HAase inhibition assay was modified from that proposed by Muckenschnabela and co-workers (284). A stock solution of 5 mg/mL HA was prepared in H_2O and stored at 4 °C. HA stock solution (50 µL) and 100 µL of buffer (0.2 M sodium formate, 0.1 M NaCl and 0.2 mg/mL BSA, pH adjusted to 3.68 with formic acid) were added to 200 µL of H_2O. Sample serial dilutions were prepared in H_2O and 50 µL of each dilution was added to each reaction tube. The reaction was started by the addition of 50 µL of HAase (600 U/mL, prepared in NaCl 0.9%), which begins the degradation of HA into n-acetylglucosamine and glucuronic acid. The enzymatic reaction was stopped by adding 25 µL of an alkaline solution consisting of di-sodium tetraborate (0.8 M in H_2O) and subsequent heating for 3 min in a boiling water bath (Morgan-Elson reaction) (**Figure 26**) (284, 285).

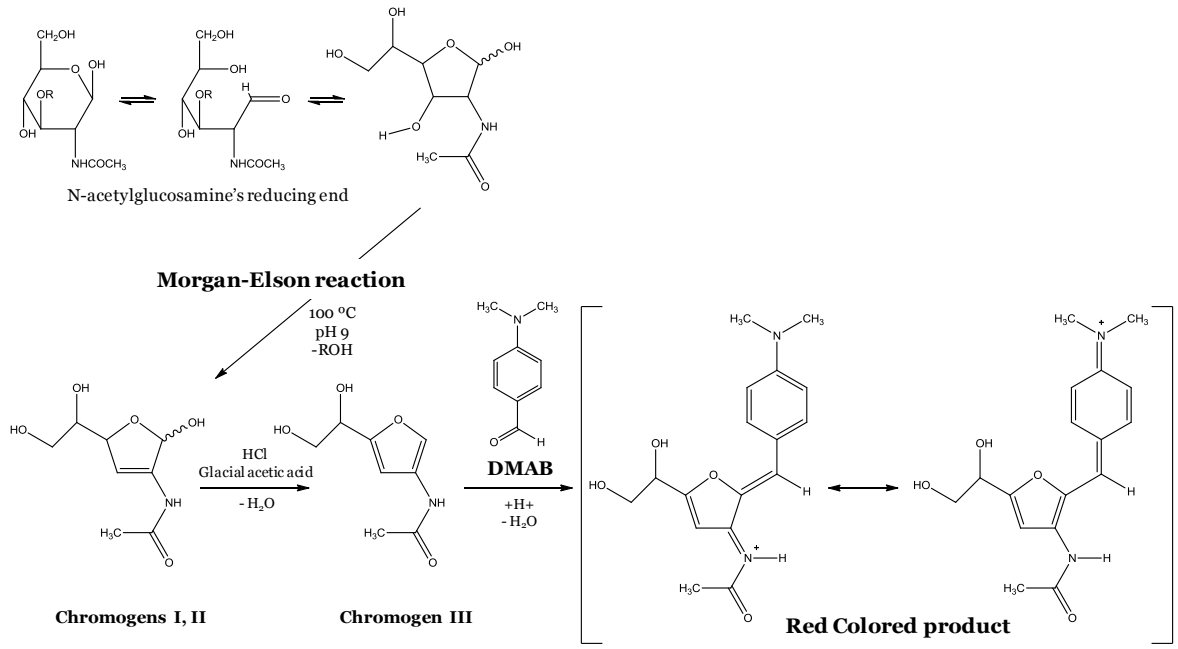

Figure 26. Morgan-Elson reaction. *(adapted from (284)).*

The test tubes were cooled at room temperature and 750 μL of DMAB solution was added (2 g of DMAB dissolved in a mixture of 2.5 mL of 10 N HCl and 17.5 mL of glacial acetic acid and further diluted 1:2 with glacial acetic acid immediately before use). After the addition of DMAB solution, the tubes were incubated at 37 °C for 20 min. The Abs of the colored product (**Figure 26**) was measured at 560 nm in a Multiskan Ascent plate reader. Three independent assays were performed in triplicate.

4.5.4. Antimicrobial activity

The antimicrobial activity of purified phlorotannins extracts was screened against nine bacteria and three fungi strains (one yeast, one dermatophyte and one *Aspergillus*). Attending to the scarcity of studies concerning the antifungal activity of phlorotannins, the most promising extracts were further evaluated against a wider range of human and animal pathogenic fungi.

4.5.4.1. Microorganisms

The study included five Gram$^+$ (*Staphylococcus aureus* ATCC 20231, *Staphylococcus epidermidis* ATCC 20044, *Micrococcus luteus* ATCC 20030, *E. faecalis* ATCC 20477 and *Bacillus cereus* ATCC 31) and four Gram$^-$ (*Salmonella typhimurium* ATCC 439 71, *Proteus mirabilis* ATCC 4479, *E. coli* ATCC30083, *Pseudomonas aeruginosa* ATCC 50071) bacteria species.

The fungi specimens comprised eight yeasts [four clinical strains of *Candida* isolated from recurrent cases of oral candidiasis (*C. albicans* D1, *C. albicans* D5, *Candida glabrata* D10R and *Candida dubliniensis* CD1) and four *Candida* reference strains (*C. albicans* ATCC 10231, *Candida krusei* ATCC 6258, *Candida tropicalis* ATCC 13803 and *Candida parapsilosis* ATCC 90018), three *Aspergillus* strains (*Aspergillus niger* ATCC 16404, *Aspergillus fumigatus* ATCC 46645 and *Aspergillus flavus* F44) and five clinical strains of dermatophytes isolated from nails and skin (*Epidermophyton floccosum* FF9, *Trichophyton rubrum* FF5, *Trichophyton mentagrophytes* FF7, *Microsporum canis* FF1 and *Microsporum gypseum* FF3).

Cultures were obtained from the Department of Microbiology, Faculty of Pharmacy, Porto University (Portugal). *C. krusei* ATCC 6258 and *S. aureus* ATCC 25923 were used for quality control in antifungal and antibacterial assays, respectively. All microorganisms were stored in broth medium with 20% glycerol at −70 °C and sub-cultured in SDA for fungi and in MHA for bacteria before each test, to ensure optimal growth conditions and purity.

4.5.4.2. Antibacterial activity

The minimum inhibitory concentration (MIC) of each extract was determined by broth microdilution methods based on the Clinical an Laboratory Standards Institute (CLSI) guidelines, reference documents M07-A8 and M100-S19, with minor modification (286). Briefly, the suspensions of bacteria cultures were prepared (from a fresh overnight culture in MHB) in ampoules containing 2 mL of NaCl 0.85% suspension medium (Api®, Biomérieux, Marcy l'Étoile, France). The suspensions turbidity was adjusted to 0.5 McFarland (using a densitometer) and then diluted in MHB till the final bacterial density of 1.5 x 10^6 colony forming units (CFU)/mL. The MIC of purified phlorotannins extracts was determined by two-fold serial dilution method, in sterile 96-wells plates (**Figure 27**).

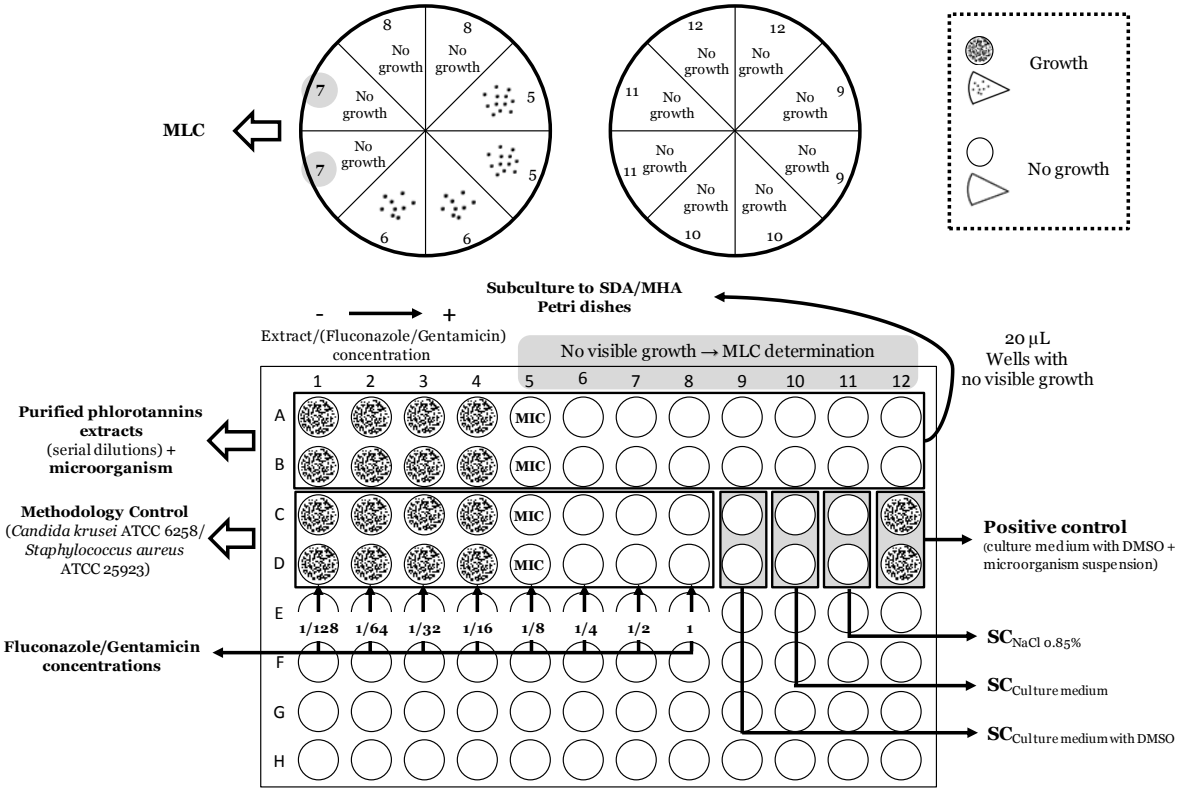

Figure 27. Schematic representation of the micromethod for the evaluation of the MIC and MLC of phlorotannins extracts, for fungi and bacteria. $SC_{NaCl\ 0.85\%}$ - Sterility control of the 0.85% NaCl solution; $SC_{culture\ medium}$ – Sterility control of the culture medium (SDB for fungi and MHB for bacteria); $SC_{culture\ medium\ with\ DMSO}$ – Sterility control of the culture medium with 2.5% DMSO; SDA – Saboraud dextrose agar; MHA – Muller Hinton agar; MIC – minimum inhibitory concentration; MLC – minimum lethal concentration.

The initial concentration was 31.3 mg/mL dry weight, for each tested species. 50 µL of the bacterial suspension was added in each well, which contained 50 µL of purified

phlorotannins extract dilutions (in MHB culture medium). The maximum DMSO concentration did not exceed 2.5% (v/v). The plates were incubated at 37 ºC in a humidified atmosphere, without agitation, for 18–24 h for both $Gram^+$ and $Gram^-$ bacteria. The MIC was determined as the lowest concentration of purified phlorotannins extracts resulting in 100% of visible growth inhibition. Gentamicin MIC for *S. aureus* ATCC 25923 was determined as quality control and the result was within the recommended limits (286). Sterility and positive controls in MHB medium alone and with 2.5% of DMSO (v/v) were included. Positive control wells contained microorganisms without antibiotics or extract. The experiments were performed in duplicate and repeated independently three times.

4.5.4.3. Antifungal activity

Broth microdilution methods based on the CLSI reference documents M27A-3 and M38-A2 for yeast and filamentous fungi, respectively, with minor modifications, were used to determine the MIC of purified phlorotannins extracts (287, 288). Briefly, cell or spore suspensions were prepared from recent cultures of the different strains of fungi on SDA with chloramphenicol and diluted to final inoculum of 10^3 CFU/mL with RPMI-1640 broth (buffered to pH 7.0 with MOPS). The MIC of purified phlorotannins extracts was determined by two-fold serial dilution method. Dilutions were prepared in RPMI-1640 broth, starting from 62.5 mg/mL dry weight for each extract. The solutions and cell suspensions in the test medium were then distributed into sterile 96-wells plates (**Figure 27**). Controls were tested along with the samples, with maximum DMSO concentrations not exceeding 2.5% (v/v). The plates were incubated in humid atmosphere, without agitation, at 35 °C (for *Candida* spp. and *Aspergillus* spp.) or 25 °C (for dermatophytes), during 48 h (for *Candida* spp.), 72 h (for *Aspergillus* spp.) or 5 days (for dermatophytes). MICs were recorded as the lowest concentrations resulting in 100% of visible growth inhibition. Fluconazole MIC for *C. krusei* ATCC 6258 was determined as quality control, and the result was within the recommended limits (287). Sterility and growth controls in RPMI-1640 medium alone and with 2.5% of DMSO (v/v) were included.

4.5.4.4. Determination of the minimum lethal concentration (MLC)

MLC of purified phlorotannins extracts was assessed for the tested bacteria and fungi. The MLC was determined after 18–24 h (for both $Gram^+$ and $Gram^-$ bacteria), 48 h (for yeasts) and 96 h (for dermatophytes) of incubation, by the subculture of an aliquot of each well of the 96-wells plates showing no visible growth (**Figure 27**). Briefly, under aseptic conditions, the content of each well with no visible growth was homogenized and

20 µL were transferred to Petri dishes containing SDA (for fungi) or MHA (for bacteria). The Petri dishes were incubated at 37 ºC for bacteria, at 35 ºC for yeasts and at 30 ºC for dermatophytes. The MLC was defined as the lowest concentration showing 100% growth inhibition in the Petri dish (**Figure 27**).

4.5.4.5. Mechanism of antifungal action of purified phlorotannins extracts

4.5.4.5.1. Influence in *C. albicans* virulence factors

The inhibition of the germ tube formation in yeast and the reduction of yeasts adhesion to the target epithelial cells have a key role in the progression of fungal infections (265, 266). The capacity of purified phlorotannins extracts from the most active seaweeds species (*C. nodicaulis*, *C. usneoides* and *F. spiralis*) was studied in the reduction of both *C. albicans* virulence factors.

Germ tube/dimorphic transition inhibition assay

Germ tube inhibition assay was performed according to Pinto and co-workers (289), with minor modifications. Purified phlorotannins extracts were dissolved in H_2O with 10% DMSO and the solution was sterilized by filtration through a 0.22 µm pore size membrane. 250 µL of each extract dilution was added to the same volume of yeast suspension, to obtain appropriate sub-inhibitory concentrations (1/2–1/256 of the MIC). After 3 h of incubation in NYP medium [N-acetylglucosamine (10^{-3} mol/L), Yeast Nitrogen Base (3.35 g/L) and proline (10^{-3} mol/L)] with NaCl (4.5 g/L, pH 6.7 ± 0.1) at 35 ºC (with shaking), 100 cells from each sample were counted using a hemocytometer and the percentage of germ tubes was determined. Germ tubes were considered positive when they were, at least, as long as the diameter of the blastospore and negative when showing a constriction at the point of connection to the mother cell, typical for pseudohyphae (**Figure 19**). The assay was performed in duplicate and repeated independently three times for each *Candida* strain.

Adherence to epithelial cells

Cells preparation and adherence assays were modified from those proposed by Lima-Neto and co-workers (290), as follows. Yeast cells were grown on SDA for 24 h at 35 ºC and re-suspended in 2 mL of sterile DPBS (pH 6.8), washed twice by centrifugation (1000 rpm during 5 min, in a Rotofix 32 A Hettich centrifuge) with 2 mL of DPBS and finally re-suspended in NYP (2 x 10^7 cells/mL). Epithelial cells were donated by the author

via soft scraping of the cheek mucous membrane with sterile cotton swabs and gently stirred and washed with DPBS by centrifugation (under the same conditions as before). Adherence assays were developed by mixing 1 mL of each suspension in a test tube, followed by incubation of the mixture in the presence of the test compounds, at 35 ºC, under gentle stirring for 2 h. A control without test compounds and a control with epithelial cells pre-treated with the test compounds were performed along with the samples. After incubation, two drops of trypan blue solution (0.4%) were added to each tube and the mixture was gently shaken. Ten µL of the stained suspension were transferred to a Neubauer chamber and examined under light microscopy.

4.5.4.5.2. Influence in fungal membrane and cell wall composition

Being the main targets for the action of antifungal drugs (156), the cell wall and cell membrane composition was evaluated in microorganisms treated with purified phlorotannins extracts from the species *C. nodicaulis*, *C. usneoides* and *F. spiralis*. *C. albicans* ATCC 10231 and *T. rubrum* FF5 were used for this assay, as the model yeast and dermatophyte organisms, respectively.

Quantification of the cell membrane ergosterol

Fungi growth conditions for sterol extraction were performed according to Pinto and co-workers (291) with minor modifications. Cell suspensions (10 µL for yeast and 50 µL for dermathophyte) were inoculated in 5 mL of RPMI-1640 medium containing different concentrations of purified phlorotannins extracts, along with a positive control (with fluconazole) and a negative control (without test extract). Cultures were incubated with shaking at 35 ºC (for *Candida* species) during 48 h and at 25 ºC (for dermatophyte) during 5 days. After the incubation period with purified phlorotannins extracts, total intracellular sterols were extracted by saponification, under the same procedure used before for the extraction of sterols from seaweeds material (Section 4.3.1). Briefly, fungal cells were harvested by centrifugation (1000 rpm during 5 min), washed, dried and weighted. Cell pellets were transferred to borosilicate glass screw-cap tubes and 3 mL of 25% alcoholic KOH solution were added to each tube, followed by a vigorous vortex agitation. Cell suspensions were incubated in a water bath at 85 ºC during 60 min. After cooling at room temperature, sterols were extracted with n-hexane and the organic phase was transferred to clean glass tubes and evaporated to dryness under nitrogen. The extracted sterols were redissolved in 0.5 mL of MeOH and analyzed by HPLC-DAD as before. Ergosterol quantification was achieved by the Abs recorded in the chromatograms,

relative to the external standard, at 280 nm. All the experiments were repeated independently three times.

Quantification of the cell wall β-D-(1→3)-glucans

Cell wall β-D-(1→3)-glucans content was determined using the aniline blue assay (292). Cell suspensions [10 µL for yeast (turbidity adjusted at 0.5 MFA) and 50 µL for dermathophyte (10^6 spores/mL in NaCl 0.85 %)] were inoculated in 10 mL of RPMI-1640 medium containing different concentrations of purified phlorotannins extracts. Positive (caspofungin) and negative (without test extract) controls were assayed along with the samples. Cultures were incubated with shaking at 35 ºC (for *Candida* species) during 48 h and at 25 ºC (for dermatophyte) during 5 days. Hyphae and yeast cells were harvested by centrifugation, washed with 0.1 M NaOH and lyophilized overnight. Five milligrams of lyophilized material were resuspended in 250 µL of NaOH (1 M) and sonicated for 30 s. The mixture was incubated (30 min at 52 ºC) and then transferred to ice (5 min). 50 µL of each reaction tube were transferred to a 96-wells plate, in triplicate, and 185 µL of aniline blue solution (0.067% aniline blue, 0.35N HCl, 0.98 M glycine in NaOH 1M, adjusted to pH 9.5) was added to each well. The microplate was incubated (30 min at 52 ºC, under light protection) and then left to cool down to room temperature. Fluorescence (*F*) readings were acquired on a fluorescence reader (SynergyTM HT, Biotek Instruments, Winooski, USA) operated by Gen5 software, with 485/20 nm excitation (ex) and 528/20 nm emission (em) wavelength. A standard curve was built using a β-D-(1→3)-glucan analog (curdlan). Results (mean ± standard deviation) are expressed as g (curdlan)/100 g (dry microorganism) of three independent assays performed in duplicate (**equation 3**).

$$[F_{ex485/20\ em528/20}\ (\text{unknown}) - F_{ex485/20em528/20}\ (\text{blank})/\text{slope}] \times 0.0235 \qquad (3)$$

Quantification of the cell wall chitin

The methodology used for cell wall chitin quantification was based on the protocol proposed by Fortwendel and co-workers (292). The fungal growth conditions, treatment and tissue harvesting were the same as those proposed for β-D-(1→3)-glucans quantification. Five milligrams of lyophilized material were resuspended in 3 mL of a saturated KOH solution and incubated for 1 h at 130 ºC. After incubation the reaction tubes were left to cool down to room temperature and then 8 mL of ethanol 75 % (ice cold) were added to each tube. The tubes were vortexed until the formation of a single layer (adding H_2O if necessary) and incubated in ice for 15 min. 300 µL of a silica suspension

(13.3% in H_2O) was added to each tube, followed by centrifugation (5 min at 1000 rpm). The supernatant was rejected and the pellet washed once with 10 mL of ice cold ethanol 40% and twice with 10 mL of ice cold water. The pellet was ressuspended in 500 µL of H_2O, 500 µL of a 5 % $NaNO_2$ solution and the same volume of 5% $KHSO_4$ (both prepared in H_2O), shacked (3 times, for 15 min at 100 rpm) and centrifuged (5 min at 1000 rpm). 150 µL of the supernatant were removed, in duplicate, from each tube and transferred to a test tube containing 450 µL of H_2O. A NH_4 sulfamate solution (12.5% m/v in H_2O) was added to each tube, followed by vortexing (1 min, 5 times). 200 µL of 3-methylbenzthiazolinone-2-hydrazone solution (5 mg/mL) were added and the mixture was incubated for 3 min at 130 ºC. The tubes were left to cool down to room temperature and then 200 µL of $FeCl_3$ solution (0.83 % in H_2O) was added to each tube, which was further incubated (25 min at room temperature, under light protection).

A standard curve was built using D-(+)-glucosamine hydrochloride as the known glucosamine sample. The Abs was read at 630 nm on a plate reader. Chitin levels were reported as g (glucosamine)/100 g (dry microorganism) (**equation 4**).

$$[½\ Abs_{630}\ (unknown) - Abs_{630}\ (blank)/slope] \times 0.15 \qquad (4)$$

Final results represent the average (± standard deviation) of three independent experiments performed in duplicate.

4.5.4.5.3. Influence in fungal metabolic activity

The influence of purified phlorotannins extracts in the metabolic activity of fungi was assessed by the evaluation of the mitochondrial activity and membrane potential in the yeast *C. albicans* ATCC 10231 and in the spores of *T. rubrum* FF5.

Mitochondrial dehydrogenases activity

Mitochondrial dehydrogenase activity was evaluated by the MTT assay. Briefly, *C. albicans* ATCC 10231 cell suspensions were prepared in ampoules containing 2 mL of NaCl 0.85% (Api®, Biomérieux, Marcy l'É toile, France) and the turbidity was adjusted to 0.5 MFA. Cell suspension dilutions (1:50 followed by 1:20) were prepared with RPMI culture medium. 500 µL of RPMI were added to the same volume of the last yeast dilution, in a 12-wells plate, and incubated overnight (18–24 h at 35 ºC). After the incubation period, cells were carefully homogenized, transferred to eppendorfs and centrifuged at 1000 rpm for 5 min. The supernatant was removed and 1 mL of the extract serial dilutions

was added to each eppendorf to obtain the appropriate concentrations (MIC to 1/1024 of the MIC). The mixture was homogenized, transferred to 12-wells plates and exposed to the purified phlorotannins extract during 1 h at 35 ºC.

For *T. rubrum*, spore suspensions were prepared in 0.85% NaCl solution, and adjusted to obtain 2.0 x 10^6 spores/mL. 1 mL of the suspension was centrifuged (1000 rpm during 5 min) and the supernatant was eliminated. 1 mL of phlorotannins serial dilutions was mixed with the pellet, homogenised and left incubating overnight at 25 ºC with shaking. After the exposure time, cell suspensions were centrifuged (1000 rpm during 5 min), the supernatant was removed and 500 mL of MTT solution (0.5 mg/mL in RPMI, 35 ºC) were added to the cell pellets and left incubating for 30 min at 35 ºC (for *Candida*) or 25 ºC (for *T. rubrum*). The insoluble purple formazan product resulting from the conversion of MTT by mitochondrial dehydrogenases of metabolically active cells (**Figure 23**) was then solubilized with 300 µL of DMSO. The extent of the reduction to formazan within the cells was quantified by measuring the Abs at 510 nm in a plate reader. Results from three independent assays performed in duplicate were expressed as the percent change of MTT reduction, using the untreated cells as control.

Mitochondrial membrane potential

The mitochondrial membrane potential was evaluated by the incorporation of the fluorescent dye RHO (293). Briefly, a suspension of *C. albicans* ATCC 10231 from an overnight culture in SDA was prepared in DPBS and the turbidity adjusted to 2.0 MFA. 880 µL of the cell suspension were mixed with 120 µL of the purified phlorotannins extracts to the desired concentrations (MIC to 1/1024 of the MIC). For controls, purified phlorotannins extracts were replaced by DPBS. After the incubation period (35 ºC during 30 min), 5 mL of 0.5 mM solution of RHO (in DMSO) were added and incubated again for 10 minutes at 35 ºC. The exceeding RHO was removed by centrifugation, for 5 min at 1000 rpm, and the fluorescence intensity was determined after resuspending the cell pellet in 1 mL of DPBS. Fluorescence intensity was determined in a fluorescence microplate reader equipped with Gen5 software, with excitation wavelength 485/20 nm and emission wavelength 528/20 nm. Results were expressed in % of fluorescence relative to control, for three independent assays performed in duplicate. Sodium azide (an inhibitor of the mitochondrial respiratory chain) at a final concentration of 20 mM was used for all the experiments as control.

4.6. Compounds isolation

Of the studied seaweeds, the one considered as the most promising (regarding bioactivity and nutritional aspects) was chosen for the isolation of compounds. Due to constrain concerning sample amounts, *F. spiralis* sample used for compounds isolation resulted from a new harvest in September 2012.

4.6.1. Crude extraction

The freeze dried seaweed material (dry weight 140 g) was exhaustively extracted (1 L) with MeOH at room temperature, under constant shaking (250 rpm). The procedure was repeated 3 times and the MeOH fractions were combined and evaporated under vacuum. The dried methanolic extract was redissolved in MeOH with 10% H_2O and subjected to solvent-solvent partitioning to give n-hexane, EtOAc and BuOH fractions (**Figure 28**). All the fractions were screened by HPLC-UV: analysis was performed in a Dionex UltiMate 3400 SD apparatus with a LPG-3400SD pump coupled to a photodiode array detector (DAD3000RS), with routine detection wavelengths at 235, 254, 280 and 340 nm. The chromatographic analysis was carried out on a Eurosphere-10 C18 column (125 × 4 mm, Knauer), working in gradient mode of 0.02% H_3PO_4in H_2O (A) and MeOH (B) (0–10 min, 10% B; 35 min, 100% B; 45 min, 100% B; flow rate of 1 mL/min and injection volume of 40 μL). The most promising fraction (ETOAc) was chosen for compounds isolation (**Figure 28**).

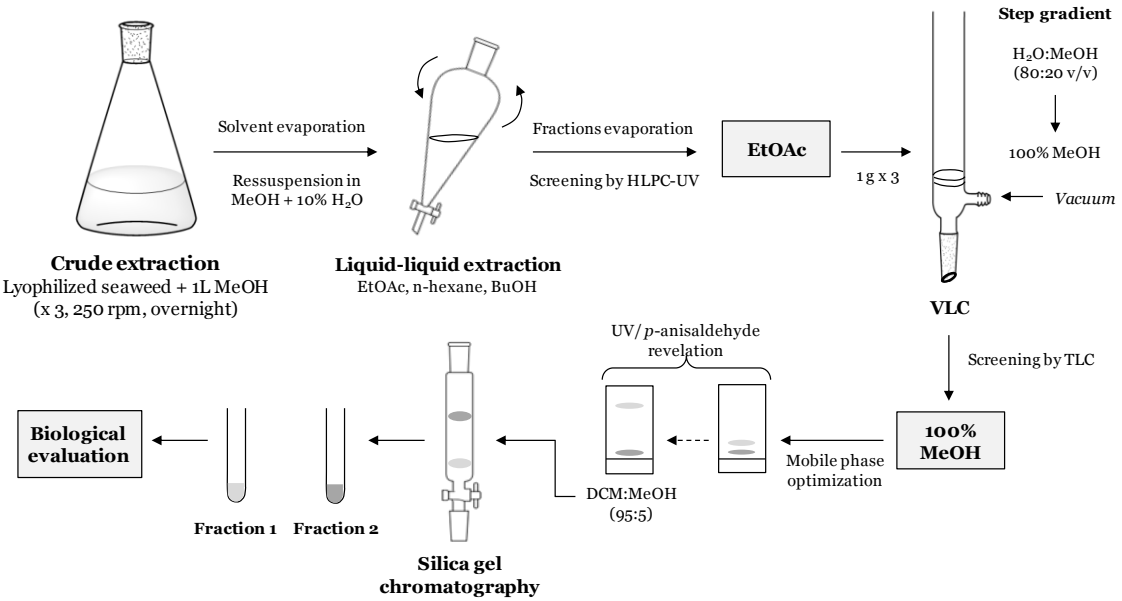

Figure 28. Schematic representation of compounds isolation from the crude extract of *F. spiralis*. EtOAc - ethyl acetate; BuOH – n-butanol; VLC – vacuum liquid chromatography; TLC – thin-layer chromatography; DCM – dichloromethane.

4.6.2. Vacuum liquid chromatography

The EtOAc fraction was evaporated to dryness, yielding 15.03 g of dry mass. Of this, 1 g was taken and was subjected to C18 reversed VLC (with silica gel 60 M), using a step gradient starting with H_2O:MeOH (80:20 v/v), followed by a final elution with 100% MeOH. This procedure was repeated 3 times, resulting in a VLC separation of a total of 3 g of the ETOAc fraction. The fractions resulting from the VLC were monitored by TLC under UV light and by spraying the plates with *p*-anisaldehyde reagent (freshly prepared 0.5 mL of *p*-anisaldehyde in 50 mL of glacial acetic acid and 1 mL of 97 % sulfuric acid, followed by heating at 105 ºC until maximum visualization of spots).

4.6.3. Isolation by silica gel chromatography

The non-polar fraction (100% MeOH) was taken and the best mobile phase was chosen using TLC plates pre-coated with silica gel 60 M F_{254}. After selecting the best mobile phase to separate the spots obtained, the 100% MeOH fraction was submitted to consecutive silica gel 60 M column chromatography with the optimized mobile phase DCM:MeOH (95:5 v/v). The isolation by silica yielded 6.09 mg of an isolated compound and 50.14 mg of an inseparable mixture of two compounds with the approximate ratio of 1:1. Both fractions were evaluated for their anti-inflammatory activity in RAW 264.7 cells, following the procedure proposed for purified phlorotannins extracts (Section 4.5.1) and using the anti-inflammatory drug dexamethasone as control.

4.7. Statistical analysis

Data were analyzed by GraphPad Prism software (version 5.02 for Windows), using the one way analysis of variance (ANOVA).

Bonferroni's multiple comparison test was carried out for sterols, on data obtained from triplicate determinations of each sample, at $P<0.05$, $P<0.01$ and $P<0.001$. Dunnett Multiple Comparison test was carried out for the determination of phlorotannins and glycerolipids anti-inflammatory activity, being used in the analysis of NO concentration in cell culture medium and in the cell-free system, and for the determination of phlorotannins toxicity by the MTT and LDH assays, from data obtained from four assays performed in duplicate for each sample, at $P<0.05$, $P<0.01$ and $P<0.001$. The same test, with the same levels of significance, was used for the determination of ergosterol, β-D-(1→3)-glucans and chitin on yeasts and dermatophytes, and on the determination of the microorganisms metabolic activity by the MTT, LHD and RHO assays, on data obtained from three independent assays performed in duplicate for each sample LPO, $O_2^{\bullet-}$ and

HAase inhibition data obtained with purified phlorotannins extracts were analyzed by Turkey's multiple comparison test, from triplicate determinations of each sample, at $P<0.05$.

PART III

RESULTS AND DISCUSSION

5. RESULTS AND DISCUSSION

5.1. Sterols profile of seaweeds samples

5.1.1. Analysis by HPLC-DAD

Most of the existing methodologies for sterols analysis deals with complex methods, including purification or isolation and derivatization of the samples for GC analysis (78). The present procedure was based on an alkaline hydrolysis of the lyophilized biomass, followed by extraction and concentration for HPLC-DAD analysis. As demonstrated herein, the proposed method is sensitive, gives good repeatability and is short in analysis time, as all the compounds are determined in less than 20 min. (**Figure 29**).

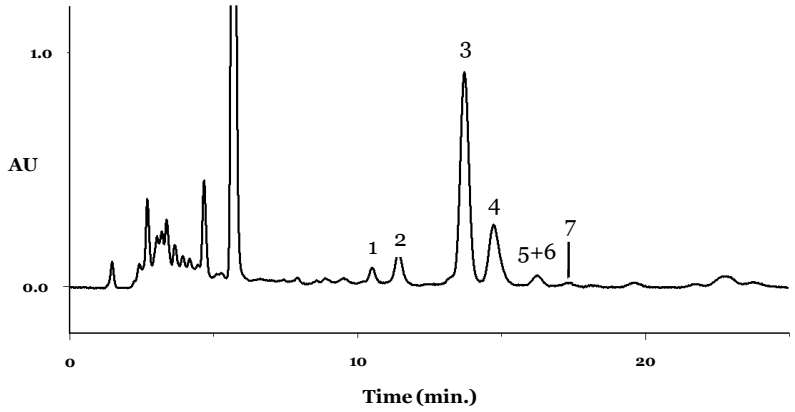

Figure 29. HPLC-DAD chromatogram of the Phaeophyta *Stypocaulon scoparium* (detection at 205 nm). Peaks: desmosterol (1), ergosterol (2), fucosterol (3), cholesterol (4), campesterol (5), stigmasterol (6) and β-sitosterol (7) (294).

The HPLC-DAD analysis of the non-saponifiable fraction of macroalgae samples allowed the determination of 7 compounds: desmosterol, ergosterol, fucosterol, cholesterol, campesterol, stigmasterol and β-sitosterol (294). In a general way, and according to previous works (295), C_{29} sterols (campesterol, ergosterol fucosterol/isofucosterol, β-sitosterol and stigmasterol) were the major group in Phaeophyta and Chlorophyta. On the other hand, C_{27} sterols (cholesterol and desmosterol) constituted the majority in Rhodophyta (**Figure 30**).

Contrarily to what happens in brown and red seaweeds, green seaweeds are characterized by the asymmetry of C-24 (24*S* configuration) which, being a characteristic of this phylum, difficults the sterols identification (54). *Codium tomentosum* Stackhouse and *Codium adhaerens* C. Agardh have been reported to contain 28-isofucosterol (54). As it happens in *Codium* spp., species of the Ulvaceae family almost always have 28-isofucosterol as the major phytosterol, while fucosterol, when present, appears in trace concentrations (48). In the present work the HPLC-DAD chromatograms of Chlorophyta

species showed an important peak with the same retention time and UV spectra of fucosterol which, according to the above mentioned, was tentatively identified as 28-isofucosterol.

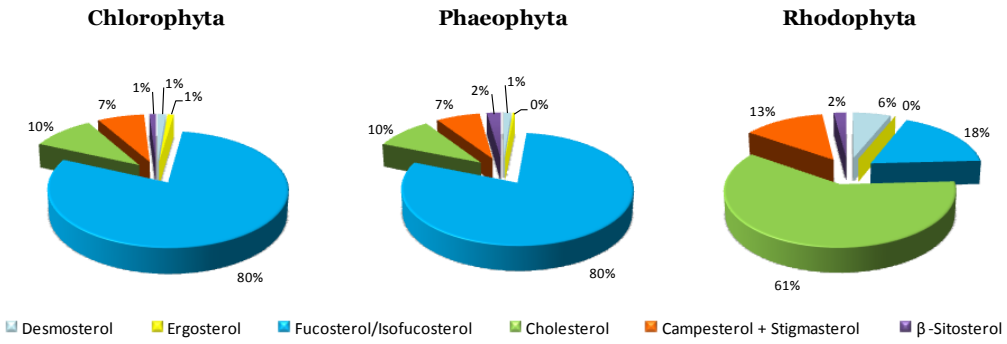

Figure 30. Sterols profile of Chlorophyta, Phaeophyta and Rhodophyta from the Portuguese west coast. Results are presented as percentage of each sterol for the species corresponding to each seaweed group (294).

Seaweeds are able to biosynthesize sterols with similar structure to that of cholesterol, but containing a methyl or ethyl group at C-24. The qualitative and quantitative sterols composition of the studied seaweeds varied between the different phyla and within the same phylum (**Figure 30**). In each phylum the species with highest sterols amounts were *C. adhaerens*, *O. pinnatifida* and *C. tamariscifolia*, this last species being the one with the highest content of sterols among all of the studied samples (294) (**Table 3**).

The sterols composition of seaweeds can be useful in taxonomic classification. Fucosterol is the dominant sterol of Phaeophyta (54), which is in agreement with the results of this study, in which all Phaeophyta contained more than 70% of fucosterol (**Figure 30, Table 3**) (294). In this phylum, the ratio fucosterol:cholesterol is usually high, although some exceptions can occur (296). Data on the chemical composition of *Padina* spp. reflect different phytosterols profiles within the genus. Although fucosterol is the main phytosterol in *P. pavonica* (297), some differences in the ratio of fucosterol to cholesterol can occur. In the analyzed sample (from the Atlantic Ocean) this ratio was 7:1, while in previous studies with a sample from the Aegean Sea (298) it was 0.3:1, 0.7:1 in a sample from the Adriatic Sea (297) and 16:1 in another from the French Mediterranean Sea (299). As expected due to its proximity, the ratio found in this study for *P. pavonica* from the Atlantic Sea is more similar to that of the algae from the Mediterranean Sea. In canned *S. polyshides* fucosterol content has been reported to be around 88% (79), while in our sample it represented 85%. The case of fucosterol in *S. Polyshides* is a valuable indicator of a kind of processing that does not affect the metabolites profile.

Table 3. Quantification of sterols (mg/Kg, dry weight)[1] by HPLC-DAD, in seaweeds samples from the phyla Chlorophyta, Phaeophyta and Rhodophyta (294).

Sample	Desmosterol	Ergosterol	Fucosterol[2]	Cholesterol	Campesterol + Stigmasterol	β -Sitosterol	Total
Chlorophyta							
U. lactuca	21.5 (0.7)^{a}	nq^{a}	2521.8 (69.9)^{a}	435.5 (29.3)^{a}	292.6 (6.6)^{a}	62.3 (1.9)^{a}	3333.7
C. adhaerens	116.1 (7.1)^{b}	147.0 (0.3)^{b}	4407.9 (24.7)^{b}	417.5 (6.1)^{a}	257.4 (8.8)$^{a, e}$	30.8 (2.8)^{b}	5376.8
C. tomentosum	49.8 (3.1)^{c}	11.1 (0.5)^{c}	3708.0 (115.5)^{c}	407.0 (7.4)^{a}	316.5 (0.7)^{a}	-^{c}	4492.5
Phaeophyta							
C. usneoides	28.9 (2.5)$^{a, e}$	-^{a}	4128.3 (18.3)^{b}	181.5 (11.9)^{b}	-^{b}	-^{c}	4338.8
C. nodicaulis	nq^{d}	-^{a}	2903.6 (22.6)^{d}	203.8 (1.9)^{b}	31.3 (1.1)$^{b, h}$	-^{c}	3138.7
C. tamariscifolia	44.1 (3.4)$^{c, e}$	-^{a}	5260.2 (14.9)^{e}	500.4 (2.6)^{c}	680.9 (21.4)^{c}	17.0 (0.3)^{b}	6502.6
F. spiralis	37.6 (3.8)^{e}	-^{a}	3815.1 (329.5)^{c}	325.1 (13.5)^{d}	183.4 (0.3)^{d}	-^{c}	4361.2
S. vulgare	47.2 (0.2)$^{c, e}$	5.6 (0.4)^{d}	4451.5 (16.7)^{b}	406.3 (13.2)^{a}	303.3 (18.9)^{a}	15.2 (2.8)$^{b, c}$	5229.1
P. pavonica	26.3 (1.0)^{a}	-^{a}	2076.0 (19.4)^{f}	285.0 (0.2)$^{d, e}$	283.3 (8.9)$^{a, e}$	141.7 (7.1)^{d}	2812.3
C. spongiosus	45.0 (1.4)$^{c, e}$	nq^{a}	1197.6 (27.2)^{g}	270.9 (10.6)^{e}	169.9 (11.2)^{d}	-^{c}	1683.3
S. scoparium	52.0 (0.0)^{c}	16.0 (0.1)^{e}	2001.6 (5.0)^{f}	362.1 (2.6)^{d}	244.4 (2.5)^{e}	52.0 (0.7)^{a}	2729.1
S. polyshides	20.6 (0.7)^{a}	-^{a}	4308.0 (62.0)^{b}	206.1 (0.9)^{b}	146.0 (5.0)$^{d, f}$	385.2 (11.9)^{e}	5065.9
H. filicina	nq^{d}	nq^{a}	1595.5 (50.6)^{h}	208.7 (0.1)^{b}	115.0 (4.6)^{f}	51.7 (2.0)^{a}	1970.9
Rhodophyta							
S. coronopifolius	54.0 (0.1)^{c}	-^{a}	138.8 (0.7)^{i}	340.2 (4.0)^{d}	462.9 (5.1)^{g}	-^{c}	995.9
A. armata	71.1 (0.5)^{f}	-^{a}	174.3 (11.0)^{i}	289.2 (9.6)$^{d, e}$	-^{b}	20.4 (0.3)^{b}	555.0
O. pinnatifida	109.7 (0.5)^{b}	-^{a}	321.7 (32.5)^{i}	1384.5 (35.7)^{f}	125.0 (8.5)^{f}	29.9 (0.3)^{b}	1970.8
S. dubyi	nq^{d}	-^{a}	30.1 (0.6)^{i}	601.6 (0.6)^{g}	-^{b}	49.1 (5.6)^{a}	680.8
P. cartilagineum	22.9 (0.3)^{a}	-^{a}	175.4 (3.9)^{i}	300.5 (2.0)$^{d, e}$	62.8 (2.3)^{h}	nq^{c}	561.5

[1] Results are expressed as mean (standard deviation) of three determinations. nq: not quantified. $^{a-i}$Different letters mean statistically significant differences in the same column ($P< 0.05$).
[2] Tentatively identified as isofucosterol in Chlorophyta (48).

Several factors can contribute to the different sterols profiles within the same seaweeds species, namely, the ecological differences, geographical origin and developmental stage of the collected organism. In particular, it has been reported that the salinity and temperature of sea water probably influence the differences in the phytosterol profile of Ulvaceae family (48). *U. lactuca* has been characterized by the presence mainly of 28-isofucosterol and cholesterol and lower amounts of other sterols (48). The ratio 28-isofucosterol:cholesterol was 10.8:1 in a sample from the Senegalese coast (300), 10.1:1 in a sample from the Mediterranean Sea (301) and 0.76:1 in another from the Adriatic Sea (48). In our sample this ratio was 5.8:1, thus being more close to that of the Mediterranean sample, which is in agreement with the geographical proximity with Atlantic Ocean (294).

Among the 18 studied algae fucosterol/isofucosterol ranged from 4 to 95% of total sterols contents, *C. usneoides* being the species with the higher relative content (**Table 3**). *C. tamariscifolia* presented the highest amount of fucosterol (**Table 3**). In Phaeophyta and Chlorophyta the content of these compounds was significantly higher than that found in Rhodophyta (**Figure 30, Table 3**) (294).

Rhodophyta are reported to primarily contain cholesterol, although several species exhibit large amounts of desmosterol (54). This is in marked contrast with the sterols in other algae (Chlorophyta and Phaeophyta) and in higher plants, which usually mainly contain C_{28} and/or C_{29} phytosterols (53). In our work cholesterol was the main compound in Rhodophyta, with the exception of *Sphaerococcus coronopifolius* Stackhouse, in which the pair campesterol+stigmasterol was predominant (**Table 3**) (294).

Cholesterol in Rhodophyta represented 34-88% of total sterols, *O. pinnatifida* being the species with higher amounts (**Table 3**) and *Schizymenia dubyi* (Chauvin ex Duby) J. Agardh the species with higher relative content. The relative amount of cholesterol in seaweeds belonging to this phylum was significantly higher than that of the other phyla. *S. polyshides* and *C. usneoides* were the species with lower cholesterol content (**Table 3**) (294).

The campesterol+stigmasterol content revealed large differences among the 18 algae species. This pair was not detected in only three species: *C. usneoides* (Phaeophyta), and *A. armata* and *S. dubyi* (Rhodophyta) (**Table 3**). *C. tamariscifolia* contained the highest amount of this pair of sterols (294).

Desmosterol, ergosterol and β-sitosterol were generally minor compounds. Desmosterol represented less than 5% of total sterols, with the exception of *A. armata*, in which it reached 13% (294). Although it has been previously published that *S. dubyi* contained a desmosterol to cholesterol ratio of 2.0:1 (302), in our sample cholesterol

largely predominates (0.02:1). According to literature, in Rhodophyta the quantities of desmosterol, a precursor of cholesterol, can change from almost pure desmosterol in spring to 100% cholesterol in autumn (303). As our samples were collected in the beginning of autumn, it was expected that cholesterol predominated relatively to desmosterol. The relative amount of desmosterol in Rhodophyta was significantly superior to that of the Phaeophyta (294).

Ergosterol was detected in all Chlorophyta, which can be a characteristic of this phylum. *C. adhaerens* clearly contained the highest amount of this compound (**Table 3**). Ergosterol was also detected in four Phaeophyta species (**Table 3**) and was absent in Rhodophyta. When present, β-sitosterol accounted for less than 8% of total phytosterols. *S. polyshides* exhibited the highest content of this sterol (**Table 3**) (294).

5.1.2. Sterols profile variations with seaweeds development

The macroalgae life cycle and the algae development stage at the time they are collected can be particularly important for the sterols profile. There are considerable variations in growth during the course of the seasons. Each algae phylum has got a favorable period for vegetative growth and reproduction, the accumulation of sterols probably corresponding to the rapid phase of cell growth (42). Rhodophyta achieves their maximum biomass production from March to June, levelling off in the period from June to September (304, 305). As our samples were collected in September, it could be expected a low phytosterol content in Rhodophyta algae, as these species would be in a decreasing phase of biomass production. In fact, the analyzed Rhodophyta species exhibited the lowest sterols content (**Table 3**). On the other hand, Phaeophyta and Chlorophyta achieve their maximum biomass production in summer, which can be an explanation for the higher amounts of sterols determined in the species belonging to these phyla (306). As far as we know, with the exceptions of *U. lactuca* (48, 307, 308), *F. spiralis* (50), *P. pavonica* (297), *A. armata* (309) and *S. polyshides* (79), the sterols profile of the remaining 13 species was achieved for the first time. As for the others, it was proved that environmental conditions can influence the chemical composition.

5.1.3. Seaweeds sterols as potential health promoters

Phytosterols are recognized as an important component of healthy diets and of diets designed to reduce hypercholesterolemia. Chlorophyta and Phaeophyta might be beneficial as food ingredients or dietary supplements to increase phytosterols intake and, thereby, lower cholesterol absorption. As supplement, phytosterols need to be formulated in order to achieve biological activity because they are inherently hydrophobic. A common

used method consists in an esterification with long chain fatty acids to increase their solubility in foods (67).

To take advantage of the cholesterol lowering effect, an increasing number of products with added phytosterols have become available in European Union. An intake of 1 to 3 g per day of added phytosterols lowers LDLc blood levels in about 5 to 15%. Besides phytosterols benefits, the market share for plant sterol enriched products in any product category is lower than 10%. As according to the World Health Organization, 60% of coronary heart disease and 40% of strokes are due to elevated cholesterol levels, phytosterols rich matrices can be explored in order to reduce the incidence of these diseases (72).

Although in Portugal *O. pinnatifida* is regularly consumed, taking into account the high phytosterols content of *C. tamariscifolia* and Fucales, in general, it might be interesting to consider these species to be used as supplement or in enriched phytosterols foods for individuals with high cholesterol levels.

5.2. Phlorotannins

5.2.1. Determination of total phlorotannins content

The extractive process used to obtain a rich purified phlorotannins extract guarantees the extraction of almost all free phlorotannins present in the samples (118, 121).

The unique structure of phlorotannins, not found in terrestrial plants, allows their quantification in the purified extracts by the DMBA colorimetric method, *via* the formation of triphenylmethane pigments after electrophilic substitution (124, 310). The indirect measurement of phlorotannins content, with good repeatability and high precision, is time and temperature dependent, reason for what these factors have to be strictly controlled (310).

The analyzed seaweeds presented a variable amount of phlorotannins, both among genus and among species of the same genus (**Figure 31**).

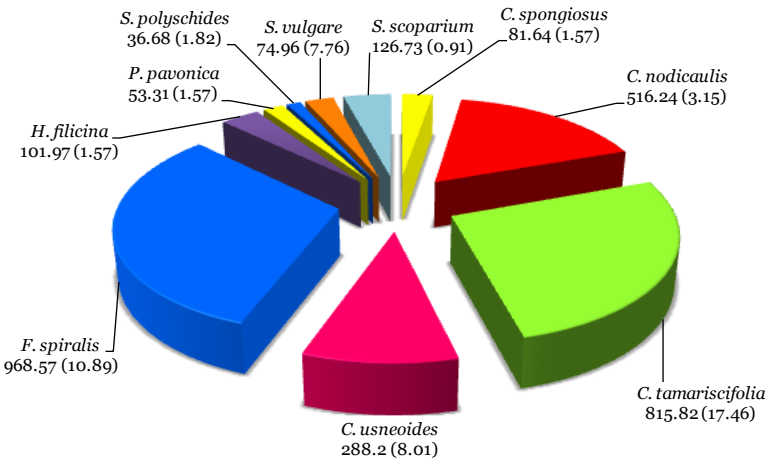

Figure 31. Phlorotannins content of brown seaweeds (mg/kg dry weight). Results are expressed as mean (standard deviation) of three determinations, expressed as phloroglucinol units (311).

S. polyshides contained the lowest amount of phlorotannins and *F. spiralis* the highest one (**Figure 31**) (311).

Previous studies confirmed that the amount of phlorotannins can be largely variable among species and may constitute up to 15 % of the dry weight of brown seaweeds (83, 84, 312). The amounts found in this work were considerably lower (<0.1 %, dry weight). The amount of cell-wall bound phlorotannins is usually much lower than the amount of soluble/free phlorotannins (118). The determination of phlorotannins content was performed in the soluble fraction of the algal matrix after an exhaustive purification technique, thus only free ones were determined in our work. However, the species and geographic origin of the samples can also explain these results: it is known that the total amount of phenolic substances in brown seaweeds in temperate seas is lower than in tropical ones (313). In the same work, the authors found that *P. pavonica* collected in the Canary Islands contained 0.69% phenolic substances, while *F. spiralis* presented 2.17% (313). Another species, *F. vesiculosus*, contained 14.5% of phlorotannins in ethanolic crude extract using the DMBA method (310).

In this survey, the phlorotannins amounts of Sargassaceae family (represented by *C. nodicaulis*, *C. usneoides*, *C. tamariscifolia* and *S. vulgare*) widely varied, *S. vulgare* being the one containing lower amounts of phlorotannins when compared with the remaining species. Phlorotannins belonging to fuhalols and fucophlorethols classes are described as the main components of this family (314). In this work the total phlorotannins amount found in the purified extracts of each family was assessed for the first time and revealed to be significantly different: the Fucaceae rank on the top while the Phyllariaceae rank at the bottom (311).

5.2.2. Anti-inflammatory potential of purified phlorotannins extracts

The anti-inflammatory potential of purified phlorotannins extracts was evaluated on the macrophage cell line RAW 264.7. Prior to testing the samples for their anti-inflammatory activities the cytotoxicity of purified phlorotannins extracts was assessed by using MTT and LDH assays. The anti-inflammatory activity of phlorotannins was screened by the determination of the extracts capacity to reduce the NO present in the supernatant of the cell culture medium.

5.2.2.1. Cytotoxicity of phlorotannins extracts

Purified phlorotannins extracts did not reveal cytotoxicity under the tested concentrations (0.5 to 8.35 mg/mL, dry weight) (311). Being non-toxic in cell systems, it is leaved opened the possibility of using these extracts in topic pharmacological formulations with anti-inflammatory properties.

The reduction of MTT varied between 92 and 107% of control (data not shown), evidencing that cells were metabolically active. LDH assay indicated cells viability between 89 and 107% (data not shown). Cells maintained their normal size and shape before and after the incubation period with the extracts. The effect of *F. spiralis* extract could not be evaluated in this cell system under the experimental conditions, due to the precipitation of the extract (solubility below 0.52 mg/mL). In fact, in order to test the same concentrations for all seaweeds, *F. spiralis* extract needed the addition of high percentages of DMSO to completely solubilize. High concentrations of DMSO are not advisable in cell systems (maximum 0.5%). In order to obey this requirement, the sample had to be much diluted and, under those conditions, no effect was observed (311).

Previous works have evaluated the effect of phlorotannins from Fucales on different cell lines. Phlorotannins rich extracts of *F. vesiculosus* were shown to lack relevant toxic effects in a rat model (113). Phlorotannins isolated from *Ecklonia cava* Kjellman (7-phloroeckol, 6,6′-bieckol, phloroglucinol, eckol, fucodiphlorethol, phlorofucofuroeckol A and dieckol) also proved to have no cytotoxicity up to 100 μM in different cell lines (MRC-5, RAW264.7 and HL-60) (315).

5.2.2.2. Effect on NO in cell culture

Purified phlorotannins extracts of most of the tested species were able to significantly reduce the levels of NO in LPS-exposed macrophage cells, at non-cytotoxic concentrations; *P. pavonica* and *S. vulgare* did not significantly reduce NO levels in comparison with control cells (**Figure 32**) (311).

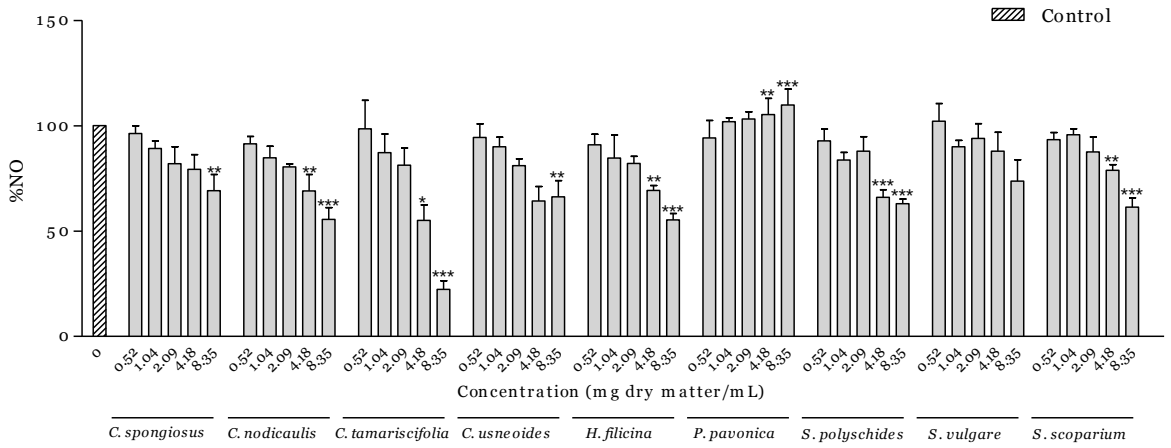

Figure 32. Effect of purified phlorotannins extracts on LPS-induced NO production in RAW 264.7 cells. Values of each group are expressed as % of LPS-only exposed cells (mean ± SEM of 4 independent assays). **P*<0.05, ***P*<0.01, ****P*<0.001 (311).

C. tamariscifolia displayed a remarkable potential for reducing NO production (**Figure 32**). At the highest concentration, NO production was reduced to 25% (*P*<0.001) of control cells. *C. nodicaulis*, *Halopteris filicina* (Grateloup) Kützing and *S. scoparium* were the species with highest activity: *C. nodicaulis* and *H. filicina* lead to a decrease of NO production to 55% and *S. scoparium* to 61% for the highest concentration (*P*<0.001) (**Figure 32**). These results may turn seaweeds phlorotannins extracts attractive potential anti-inflammatory agents or co-adjuvants in the treatment of inflammatory states. The inhibitory effect of the extracts on NO production cannot be attributed to cytotoxic effects, since they did not decrease cell viability, as discussed above (311).

Brown seaweeds can exert anti-inflammatory activity by diverse processes, not necessarily related to the decrease of NO production. For example, anti-inflammatory effect *via* the arachidonic dependent pathway (down regulation of prostaglandin E2 generation) was observed for a phlorotannins extract of *E. cava* (112). Isolated phlorotannins have also shown to inhibit lipoxygenases and phospholipase A_2 activity (114). In another assay moderate Cox-1 inhibition by several phlorotannins was observed,

which may contribute for their anti-inflammatory capacity (211). Phlorotannins can affect NO levels by direct scavenging and/or by decreasing NO production through the action in the inflammatory signaling cascade or by inhibiting the enzymes involved in NO production (111, 112).

The differences observed in the anti-inflammatory activity can be related, at least partially, with the total amount of phlorotannins present in the seaweeds extracts. In what concerns to extracts of the same species but with different phlorotannins contents, the work by Zaragozá and colleagues with *F. vesiculosus* extracts (113) showed that the potential to reduce NO is proportional to the phlorotannins content. Considering the probable similarity of the qualitative composition of the three *Cystoseira* species studied herein, the results seem to be in agreement with those of Zaragozá and co-workers, as the capacity of these species to reduce NO in the culture medium was proportional to their phlorotannins content (**Figures 31** and **32**). However, the qualitative composition of the extracts may not be excluded, as species with similar phlorotannins content presented different activities (316). *S. polyschides* and *P. pavonica* are example of this: the first displayed a much higer capacity to reduce NO levels, even presenting a lower phlorotannins content (311).

5.2.2.3. Effect of NO in a cell-free system

The decrease of NO concentration can be due to the interference with NO production by LPS-stimulated macrophages or to the scavenging of the produced NO. In order to test the later hypothesis, a cell-free assay with a NO donor was performed. The concentrations of purified phlorotannins extracts tested on the cell free system were the same as those used for macrophage cells. Under these conditions, *Cladostephus spongiosus* (Hudson) C. Agardh, *H. filicina*, *S. polyschydes* and *P. pavonica* had no activity. As a first approach, it seems that phlorotannins can act by reducing NO by, at least, two different mechanisms. As far as we know, the NO scavenging activity of purified phlorotannins extracts for the ten brown seaweeds under study had not been previously assessed.

Five of the brown seaweed species (the three *Cystoseira*, *S. vulgare* and *F. spiralis*) showed significant NO scavenging activity, in a dose-dependent manner (**Figure 33**) (311).

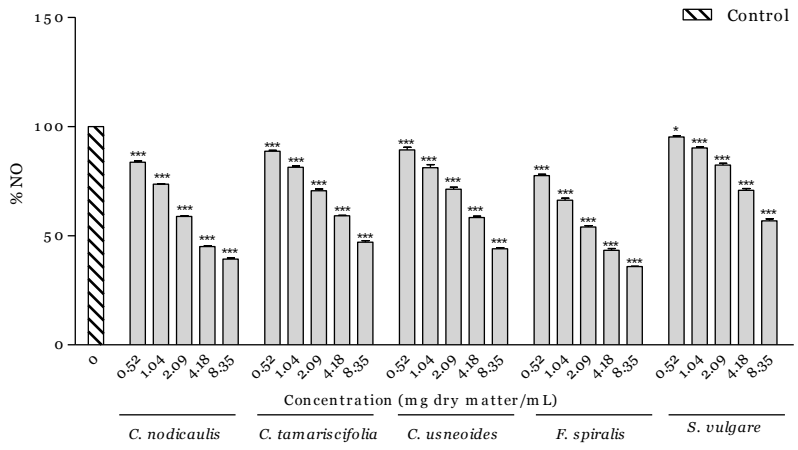

Figure 33. Effect of purified phlorotannins extracts on SNP-generated NO in a cell-free system. Values of each group are expressed as % of control (mean ± SEM of 4 independent assays). **P*<0.05, ***P*<0.01, ****P*<0.001 (311).

The decrease of NO seen in the cellular system may be partially attributed to the direct NO scavenging, as the purified phlorotannins extracts of these species decreased the amount of nitrite generated from the decomposition of SNP. In contrast to the cellular assays, in which *C. tamariscifolia* showed the highest potential to reduce NO, in the cell-free system *F. spiralis* and *C. nodicaulis* exhibited the highest capacity for NO radical scavenging, leading to a reduction of about 40% when compared with the control (**Figure 33**) (311). On the other hand, the extracts of *H. filicina*, *S. polyschides* and *S. scoparium* reduced the levels of NO on cells stimulated by LPS, having no activity in the cell-free system. The differences observed between the cellular and non-cellular assays suggest that phlorotannins can reduce NO by different mechanisms. The evaluation of NO scavenging capacity of phlorotannins in the cell-free system evidences that these compounds have the capacity to sequester NO. On the other hand, the reduction on NO levels in the culture medium of cells treated with *C. spongiosus*, *H. filicina*, *S. polyschydes* and *P. pavonica* (which have no capacity to reduce NO generated from SNP) demonstrates that there are other mechanisms responsible for NO reduction, besides scavenging. These mechanisms of decreasing NO that may be involved in the cellular assay, can be, for example, those related to the several inflammatory cascade steps (111, 112, 317). As so, the scavenging effect observed in the cell-free system with *C. nodicaulis*, *C. tamariscifolia*, *C. usneoides* and *S. vulgare* can contribute to the decrease of NO noticed in the cellular system with these species.

5.2.3. Screening of the antimicrobial activity of purified phlorotannins extracts

5.2.3.1. Antibacterial activity

The antibacterial activity of purified phlorotannins extracts of the studied species was investigated *in vitro* for the first time herein. The results obtained highlight how the species variability and their phlorotannins content can influence the antibacterial properties.

Purified extracts from the ten seaweeds were tested against several Gram$^+$ and Gram$^-$ bacterial strains (311). The values of MIC and MLC were determined and are displayed in **Table 4**. Gram$^+$ bacteria were more sensitive to phlorotannins than Gram$^-$. The purified phlorotannins extracts showed bacteriostatic activity against all the studied strains and bactericidal activity against five of them (*S. epidermidis*, *M. luteus*, *E. faecalis*, *B. cereus* and *E. coli*). There was no considerable variation in the MLC found among the different studied bacteria, with the exception of *M. luteus* treated with *F. spiralis* extract, which revealed to be the most lethal agent (**Table 4**). *S. epidermidis* was the most susceptible species among Gram$^+$, the inhibitory effect of *C. nodicaulis* extract on its growth being directly proportional to the phlorotannins extract concentration and lethal at 31.3 mg/mL (**Table 4**). In a general way, and according to the results, *F. spiralis* and *C. nodicaulis* were the most promising seaweeds (**Table 4**) (311).

Gram$^+$ bacteria

In a general way, *Cystoseira* species and *F. spiralis* were the most active against *Staphylococcus*. Of them, *S. epidermidis* was the most sensitive species, with the lowest MIC and MLC values for treatment with *F. spiralis* (**Table 4, Figure 34**) and *C. nodicaulis* (**Table 4**). *S. aureus* was also more sensitive to *C. nodicaulis* and *F. spiralis* (MIC=7.8 mg/mL) (311). These Gram$^+$ cocci are members of the Micrococcaceae family, and are more often associated with human infection, *S. aureus* being the major cause of morbidity and mortality. Among the more common infections caused by *S. aureus* are boils, folliculitis, cellulitis and impetigo, which can be a risk in immunocompromised individuals. This strain is also capable of producing food poisoning. On the other hand, *S. epidermidis* is recognized as the leading cause of nosocomial infections, being responsible for a subtle and sub-acute clinical presentation. This strain also presents the ability to form biofilms, which can be a risk in intravenous drug users (318).

Table 4. Minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) of purified phlorotannins extracts against selected bacteria (311).

Seaweed extract[1]	Gram+										Gram–							
	S. aureus		*S. epidermidis*		*M. luteus*		*E. faecalis*		*B. cereus*		*P. mirabilis*		*E. coli*		*P. aeruginosa*		*S. typhimurium*	
	MIC	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**
C. spongiosus	>31.3	-	31.3	>31.3	>31.3	-	>31.3	-	>31.3	-	>31.3	-	>31.3	-	>31.3	-	>31.3	-
C. nodicaulis	7.8	>31.3	3.9	31.3	15.6	31.3	>31.3	-	15.6	>31.3	15.6	>31.3	31.3	31.3	31.3	>31.3	31.3	>31.3
C. tamariscifolia	15.6	>31.3	7.8	>31.3	15.6	31.3	>31.3	-	15.6	>31.3	31.3	>31.3	>31.3	-	>31.3	-	>31.3	-
C. usneoides	15.6	>31.3	7.8	>31.3	31.3	>31.3	>31.3	-	31.3	>31.3	31.3	>31.3	>31.3	-	>31.3	-	>31.3	-
F. spiralis	7.8	>31.3	3.9	31.3	2.0	15.6	15.6	31.3	7.8	31.3	31.3	>31.3	>31.3	-	31.3	>31.3	>31.3	-
H. filicina	>31.3	-	31.3	>31.3	>31.3	-	>31.3	-	>31.3	-	>31.3	-	>31.3	-	>31.3	-	>31.3	-
P. pavonica	>31.3	-	15.6	>31.3	>31.3	-	>31.3	-	>31.3	-	>31.3	-	>31.3	-	>31.3	-	>31.3	-
S. polyschides	>31.3	-	15.6	>31.3	>31.3	-	>31.3	-	>31.3	-	>31.3	-	>31.3	-	>31.3	-	>31.3	-
S. vulgare	31.3	>31.3	7.8	>31.3	>31.3	-	31.3	>31.3	>31.3	-	31.3	>31.3	>31.3	-	>31.3	-	>31.3	-
S. scoparium	>31.3	-	31.3	>31.3	>31.3	-	>31.3	-	>31.3	-	>31.3	-	>31.3	-	>31.3	-	>31.3	-

[1] mg/mL (dry weight)

Of the studied seaweeds, *F. spiralis* presented the best activity against *M. luteus* (MIC=2.0 mg/mL) (**Table 4**). This seaweed also presented the highest bactericidal activity, being capable of killing *M. luteus* at 15.6 mg/mL (**Table 4, Figure 34**) (311). *M. luteus* has been implicated in a variety of infections, including meningitis, endocarditis, septic arthritis and central nervous system infections in immunocompromised hosts (318).

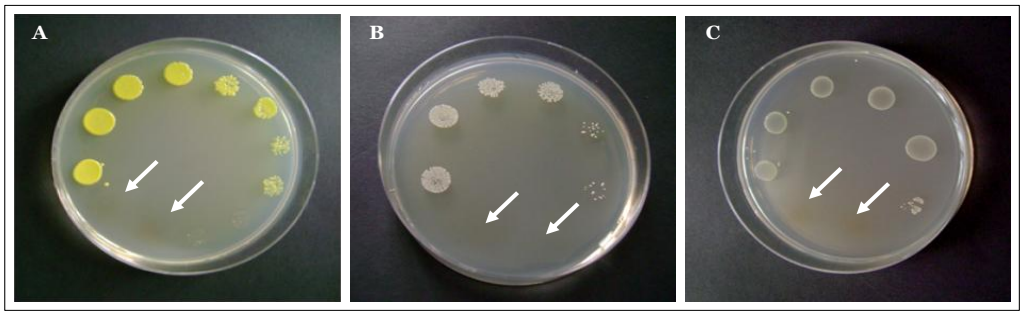

Figure 34. Minimum lethal concentration (MLC) of *F. spiralis* against *M. luteus* (A), *S. epidermidis* (B) and *E. faecalis* (C). Arrows point the area of growth inhibition corresponding to the MLC. (*Photographs of Graciliana Lopes*).

The species belonging to the genus *Cystoseira* and *Fucus* were the only ones active against *B. cereus*, with *F.spiralis* also presenting bactericidal activity (**Table 4**). *B. cereus* is an important pathogen responsible for food-borne illness, causing intoxication or infection in humans (318).

E. faecalis, which commonly colonizes gastrointestinal and genital tracts of humans, was the most resistant among Gram$^+$ bacteria. Only *F. spiralis* and *S. vulgare* were active against this microorganism and only *F. spiralis* showed bactericidal activity (**Table 4, Figure 34**). In general, species belonging to the genus *Cystoseira* and *F. spiralis* were the ones with highest bacteriostatic and bactericidal activity, which can be related with their high content in phlorotannins (311).

Gram$^-$ bacteria

Phlorotannins purified extracts evidenced lower activity against Gram$^-$ strains (**Table 4**). *C. nodicaulis* was the only seaweed with ability to inhibit *S. typhimurium* growth (**Table 4**). Most infections with *Salmonella* are caused by contaminated food and water. Of concern are *Salmonella* infections that are resistant to several antimicrobial agents, in which the serotype *Typhimurium* is included (318).

F. spiralis and *C. nodicaulis* extracts were the only ones active against *P. aeruginosa* (**Table 4**), a bacterium responsible for a wide range of infections, especially prevalent

among patients with burn wounds, cystic fibrosis, acute leukemia, organ transplants and intravenous drug addiction. It also causes urinary tract and lower respiratory tract infections, particularly severe and even life-threatening in immunosuppressed hosts (318).

P. mirabilis was the most sensitive strain studied. It was inhibited by five of the tested extracts between 15.6-31.3 mg/mL (**Table 4**) (311). *C. nodicaulis* was the most effective seaweed against this bacterium, which is normally found in human gastrointestinal tract and can cause urinary tract and wound infections and septicemia. It is also often associated with nosocomial infections (318) .

C. nodicaulis was the only seaweed capable of inhibiting *E. coli* growth, being also lethal for this bacterium at the same extract concentration (31.3 mg/mL) (**Table 4**) (311). *E. coli* is the most common member of *Escherichia* genus, normally found in the gastrointestinal tract of humans and animals. It causes gastrointestinal and extra intestinal infections and is spread by fecal-oral route or by improperly cooked or contaminated food and drinks (318) .

The physical differences between Gram$^-$ and Gram$^+$ bacteria can be the basis of the observed behavior of phlorotannins extracts. Gram$^-$ bacteria are surrounded by an external membrane with high lipopolysaccharide content. This membrane, in addition to other mechanisms, confers bacteria the capacity to resist to several antibiotics and, consequently, to the action of many natural extracts. Having this in mind, it is expectable Gram$^+$ strains to be more sensitive and, consequently, infections caused by them easier to overcome.

Nagayama and co-workers (106) concluded that the antibacterial effects of phlorotannins tend to increase with the polymerization of phloroglucinol. *S. aureus* and *B. cereus* were the only Gram$^+$ strains studied by this group. *S. aureus* was more sensitive than *B. cereus*, which is in accordance with our results. Taking this into account, it is expectable that species belonging to the genus *Cystoseira* and *Fucus* are probably the ones with phlorotannins of higher molecular weight, as they are the ones presenting lower MICs for all the studied bacteria. On the other hand, and according to the tannic compounds ability of reacting with amine groups of proteins by their free OH groups (250), it is also probable that those species with phlorotannins with a higher degree of polymerization have more free OH groups capable of reacting with the amine groups of proteins from the bacterial surface. Although the mechanism of action of phlorotannins with bacteriostatic and bactericidal activities is not yet elucidated, it is possible that there is some relation with the action of these compounds on the microorganisms surface (251).

In fact, it is thought that the interactions between bacterial proteins and phlorotannins play an important role in the bactericidal action of these compounds (251).

Concerning *Cystoseira* genus, although *C. tamariscifolia* presented the highest phlorotannins amount (**Figure 31**), the extract was less effective against bacteria than that of *C. nodicaulis* and *C. usneoides*. Thus, it is possible that polyphenols present in *C. tamariscifolia* exhibit lower molecular weight or fewer OH groups free to react when compared with those in *C. nodicaulis* and *C. usneoides* (311).

The available studies concerning the antibacterial activity of phlorotannins are, in their majority, related to phlorotannins isolated from seaweeds of the genus *Ecklonia* and *Eisenia*. Compounds like eckol, 8,8'-Bieckol, dieckol, dieckoldioxinodehydroeckol, fucofuroeckol-A, 7-phloroeckol and phlorofucofuroeckol-A have been shown to have antimicrobial activity against several bacterial strains, such as *S. aureus*, methicillin-resistant *S. aureus* (MRSA), *Salmonella* spp., *Bacillus* spp., *Campylobacter jejuni* and *E. coli*, among others (106, 108, 247, 248). The effect of phlorotannins on bacterial growth inhibition has been compared to that of catechins from green tea, used as positive control, and their activity was equal or superior to that (108).

Considering the pathogenic capability of Gram$^-$ bacteria and the emerging multidrug resistance, especially against third and fourth-generation cephalosporins and quinolones antibiotics, it becomes important the development of new antimicrobial drugs (229). Taking into account the MIC values obtained for *F. spiralis* and *C. nodicaulis*, these species appear to be promising for a future antimicrobial approach.

5.2.3.2. Antifungal activity

Results of the antifungal screening of phlorotannins extracts against the yeast *C. albicans*, the filamentous fungi *A. fumigatus* and the dermatophyte *T. rubrum* are shown in **Table 5.** Phlorotannins extracts seemed to be less effective against fungi than bacteria, in the tested concentrations. The purified extracts displayed antifungal properties against two fungi strains: *T. rubrum* and *C. albicans*, being inactive against *Aspergillus* (311).

Table 5. MIC and MLC of purified phlorotannins extracts against selected fungi (311).

Seaweed Extract[1]	Fungi					
	C. albicans		*A. fumigatus*		*T. rubrum*	
	MIC	**MLC**	**MIC**	**MLC**	**MIC**	**MLC**
C. spongiosus	>31.3	-	>31.3	-	>31.3	-
C. nodicaulis	7.8	>31.3	>31.3	-	3.9	7.8
C. tamariscifolia	>31.3	-	>31.3	-	31.3	>31.3
C. usneoides	31.3	>31.3	>31.3	-	7.8	31.3
F. spiralis	31.3	>31.3	>31.3	-	3.9	31.3
H. filicina	>31.3	-	>31.3	-	>31.3	-
P. pavonica	>31.3	-	>31.3	-	>31.3	-
S. polyschides	>31.3	-	>31.3	-	>31.3	-
S. vulgare	15.6-31.3	>31.3	>31.3	-	7.8	31.3
S. scoparium	>31.3	-	>31.3	-	>31.3	-

[1]mg/mL (dry weight)

Of the studied fungi, *T. rubrum* was the most sensitive to the purified phlorotannins extracts. *F. spiralis* and *C. nodicaulis* were the most effective species (MIC=3.9 mg/mL), followed by *C. usneoides* and *S. vulgare* (MIC=7.8 mg/mL) and *C. tamariscifolia* (31.3 mg/mL). All the other seaweed extracts were inactive under the tested concentrations (**Table 5**). The MLC was also assessed against *T. rubrum*. Best results were obtained with *C. nodicaulis*, being lethal for the fungi at 7.8 mg/mL (311). These results point *C. nodicaulis* as very promising for the future development of antimycotic drugs, since the tested phlorotannins extract acted as fungicidal (MLC=MIC or MLC=MIC-1 dilution). The importance of *C. nodicaulis* against *T. rubrum* takes a key role if one considers the therapy abandonment by patients, due to the extent of the treatment period.

Only four species displayed activity against *C. albicans* under the tested concentrations, *C. nodicaulis* being the most effective one (**Table 5**). Under the tested concentrations none of the studied species was lethal for *C. albicans* (MLC>31.3 mg/mL) (311). Nevertheless, the active seaweeds can be considered as an alternative for the treatment of disseminated candidiasis, in combined therapy with other antifungal agents. C*andida* cells can establish disease by disseminating from mucosa and gut, which is in the origin of invasive infections, particularly serious in individuals with weakened immune defenses. Since phlorotannins extracts did not reveal toxicity to mammalian cells, they can be especially interesting for the treatment of fungal infections in immunocompromised

patients, as the side effects related to the antifungal drug will be, in this situation, less expected.

In a general way, *C. nodicaulis* revealed to be the most effective species against the studied fungi. Although it was not lethal for *C. albicans* under the tested concentrations, it presented the lowest MICs for both *C. albicans* and *T. rubrum*, being also lethal for the dermatophyte (311).

According to these results, purified phlorotannins extracts can be pointed to act as antimicrobial, antioxidant and anti-inflammatory agents. Species belonging to the genus *Cystoseira* and *F. spiralis* revealed potential for the development of medicines with anti-inflammatory and antimicrobial activities, especially against $Gram^+$ strains and dermatophytes, which showed to be more sensitive to them. Additionally, the NO scavenging capacity observed in cell-free systems confirms the antioxidant potential of phlorotannins, which can contribute to the overall beneficial effects. These results point phlorotannins extracts as potential pharmaceutical resources for combating infections with multi etiological causes, as the same extract can cover a wide range of microorganisms and has the potential to act on inflammatory states and oxidative environment, commonly associated with microbial infections.

Taking into account the importance of the mechanism of action on therapeutic effectiveness of antifungals, and considering the relative scarcity of antifungal drugs comparatively to antibacterial ones and the potentialities of phlorotannins, these compounds were further evaluated on this field.

5.2.4. Mechanism of antifungal action of phlorotannins

Whereas the medicinal properties of herbs have been recognized since ancient times, there has been a resurgence of interest in the antimicrobial properties of marine organisms, particularly seaweeds. With this in mind, phlorotannins extracts from the seaweeds species that have shown most promising antifungal activity in the first screening, namely *C. nodicaulis*, *C. usneoides* and *F. spiralis*, were used for the study of the mechanism of antifungal action.

On a first approach, it has been made a screening of the antifungal activity of the phlorotannins extracts on a broader range of fungi (**Table 6**).

Table 6. Activity (MIC/MLC) of purified phlorotannins extracts from brown seaweeds against selected yeast and filamentous fungi[1] (319).

Strains	Seaweed								
	C. nodicaulis			*C. usneoides*			*F. spiralis*		
	MIC[1]	MIC_{50}	MLC[1]	MIC[1]	MIC_{50}[1]	MLC[1]	MIC[1]	MIC_{50}[1]	MLC[1]
Yeast									
ATCC									
C. albicans ATCC 10231	15.6	-	>62.5	31.3	-	>62.5	31.3	-	>62.5
C. krusei ATCC 6258	31.3	-	>62.5	31.3	-	>62.5	>62.5	-	-
C. tropicalis ATCC 13803	>62.5	-	-	>62.5	-	-	>62.5	-	-
C. parapsilosis ATCC 90018	62.5	-	>62.5	62.5	-	>62.5	>62.5	-	-
Clinical									
C. albicans D1	62.5	-	>62.5	>62.5	62.5	-	>62.5	-	-
C. albicans D5	62.5	31.3	>62.5	>62.5	62.5	>62.5	>62.5	-	-
C. glabrata D10R	>62.5	2.0	-	>62.5	-	-	>62.5	-	-
C. dubliniensis CD1	62.5	31.3	>62.5	62.5	-	>62.5	>62.5	-	-
Filamentous fungi									
Dermatophytes									
E. floccosum FF9	3.9	-	7.8	15.6	-	15.6	7.8	-	7.8
T. rubrum FF5	3.9	-	7.8	7.8	-	31.3	3.9	-	31.3
T. mentagrophytes FF7	7.8	-	7.8	31.3	-	31.3	15.6	-	15.6
M. canis FF1	31.3	-	31.3	31.3	-	>62.5	15.6	-	15.6
M. gypseum FF3	31.3	-	>62.5	>62.5	-	-	31.3	-	>62.5
Aspergillus									
A. flavus F44	>62.5	-	-	>62.5	-	-	>62.5	-	-
A. fumigatus ATCC 46645	>62.5	-	-	>62.5	-	-	>62.5	-	-
A. niger ATCC 16404	>62.5	-	-	>62.5	-	-	>62.5	-	-

[1]MIC, MIC_{50} and MLC were determined by a microdilution method and expressed in mg/mL (dry weight); "-"Not determined.

The tested extracts displayed antifungal properties against six yeast strains (*C. albicans* ATCC 10231, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 90018, *C. albicans* D1, *C. albicans* D5 and *C. dubliniensis*), *C. albicans* ATCC 10231 being the most sensitive. It was also possible to determine the MIC_{50} for *C. glabrata* in the tested concentrations (**Table 6**) (319). With the exception of *M. gypseum* that was resistant to *C. usneoides*, all of the studied dermatophytes were sensitive to the purified phlorotannins extracts, with fungistatic and fungicidal activity. *T. rubrum* and *E. floccosum* were the most sensitive, being inhibited by all the purified phlorotannins extracts. *C. nodicaulis* presented the lowest MIC and MLC value for both species, followed by *F. spiralis* (**Table 6**). Under the tested conditions, all of the studied *Aspergillus* species were resistant to the tested extracts at the concentration of 62.5 mg/mL (**Table 6**). Contrarily to *Candida* species, and with the exception of *M. gypseum*, purified phlorotannins extracts presented fungicidal activity against almost all of the studied dermatophyte strains (with MLC=MIC or MLC= at least one dilution before MIC) (319). Considering this, phlorotannins can be promising antifungals, both individually and in combined therapy.

According to the displayed antifungal activity, the purified phlorotannins extracts were object of further investigation, in an attempt to elucidate the mechanism underlying their antifungal action. The effects on the dimorphic transition and adherence (in *C. albicans*), on the fungal cell wall and membrane composition and on the mitochondrial function were addressed.

5.2.4.1. Effect on *C. albicans* virulence factors

The dimorphic transition of *C. albicans*, i.e., the ability to produce a germ tube, is involved in the microorganism pathogenesis. The inhibition of the germ tube production is sometimes sufficient to treat disseminated candidiasis, as this structure is responsible for the adhesion of *Candida* cells to the mucosa, which turns these infections more difficult to overcome (265, 266). The ability to produce a germ tube is characteristic of *C. albicans* species and represents a good tool for the presumptive identification of clinical isolates (267). With the purpose of evaluating the effect of phlorotannins on the dissemination of *Candida* cells, the study of the dimorphic transition and adherence of these cells was performed in the presence of the purified phlorotannins extracts.

5.2.4.1.1. Dimorphic transition

Of the genus *Candida*, only the species *C. albicans* and *C. dubliniensis* display the capability to undergo dimorphic transition (265, 267). In order to evaluate the effect of purified phlorotannins extracts in yeast dimorphic transition, and taking into account that *C. dubliniensis* does not produce a germ tube *in vivo*, three *C. albicans* were selected: one type strain (ATCC 10231) and two clinical isolates (D1 and D5). Purified phlorotannins extracts of species from the genus *Cystoseira* did not inhibit the germ tube formation in the tested *C. albicans* strains. However, purified phlorotannins extracts from *F. spiralis* inhibited similarly the dimorphic transition in the three *C. albicans* strains studied (**Figure 35**). More than 90% of the yeast cells treated with sublethal concentrations of purified phlorotannins extracts from *F. spiralis* (from MIC to MIC/32) presented pseudohyphae instead of germ tubes (**Figure 35 B1**), pointing this species as a potential inhibitor of *Candida* infection dissemination (265). MIC/32 was the lowest concentration for which more than 90% of yeast cells presented pseudohyphae (**Figure 35 C**). For MIC/64, the percentage of yeast cells with pseudohyphae was reduced to values around 70% and, for the lowest concentrations tested (MIC/128 and MIC/256), pseudohyphae represented less than 10%, the production of germ tubes being no longer inhibited (**Figure 35 C**).

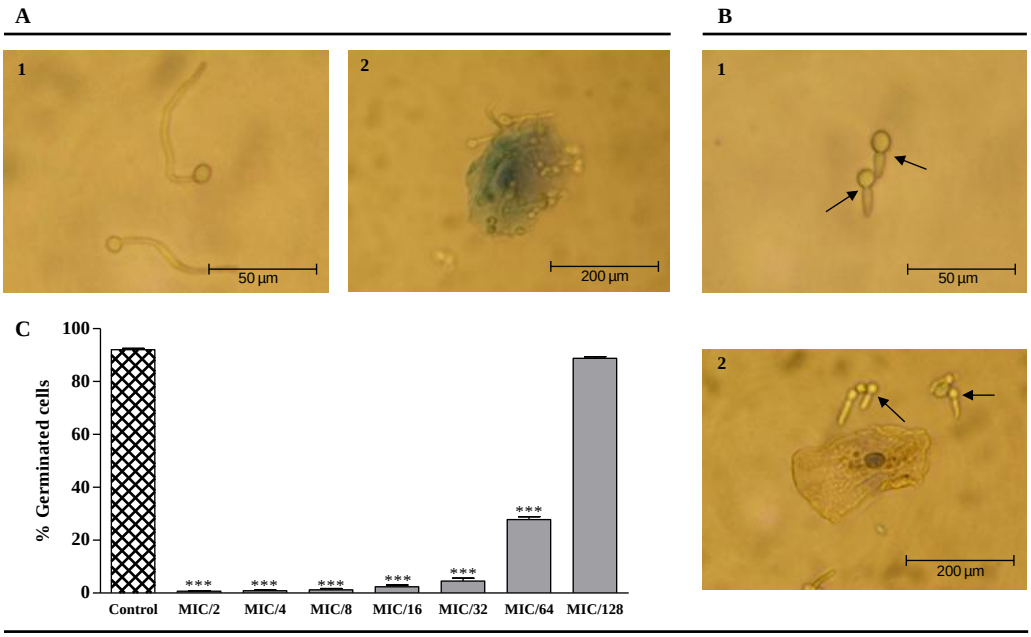

Figure 35. Effect of purified phlorotannins extracts from *F. spiralis* in the dimorphic transition of *C. albicans* ATCC 10231 (Untreated control cells - **A1**; cells treated with extract at MIC/32 – **B1**), in the adherence of the yeast to the epithelial cells (Untreated control cells – **A2**; cells treated with extract at MIC/32 - **B2**) and in the germ tube formation (**C**). Arrows show a constriction resulting from an incomplete budding, where the bud remains attached to the mother cell, originating pseudohyphae. Levels of magnification are as shown: Bars, 50 µm (A1 and B1) and 250 µm (A2 and B2). Results are expressed as mean (±SD) of three independent assays. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ (C) (319).

The results demonstrate that phlorotannins of *F. spiralis* inhibit the dimorphic transition of *Candida* cells by causing an incomplete budding, where the bud is not detached from the mother cell, originating pseudohyphae.

5.2.4.1.2. Adherence to epithelial cells

The adherence assay with epithelial cells was performed to clarify if pseudohyphae formation could affect the adherence of *Candida* to the mucosal cells. **Figure 35** shows the effect of purified phlorotannins extracts in the dimorphic transition of *C. albicans* ATCC 10231 and in the adherence of the yeast to epithelial cells, for the lowest *F. spiralis* concentration capable of inhibiting the dimorphic transition in more than 90% of yeast. A common distribution pattern was observed in control yeast cells and in yeast cells treated with phlorotannins. *C. albicans* treated with *F. spiralis* phlorotannins presented a widespread distribution through the culture medium, with few or no adherence to epithelial cells (**Figure 35 B2**). Contrarily, in control cells, yeast appeared adhered to epithelial cells and few yeast where found free in the culture medium (**Figure 35 A2**). These observations confirm the adherence capacity of *C. albicans* germ tubes and the lack of adherence for pseudohyphae, emphasizing the potential of *F. spiralis* to reduce the virulence factor in *Candida* species. This effect can be the cause of phlorotannins fungistatic activity and is also an important target concerning the virulence of these microorganisms, as this species can significantly inhibit germination until MIC/64 (**Figure 35 C**).

Hereupon, phlorotannins of *F. spiralis* seem to be promising for association with existing antifungals, possibly being beneficial for the treatment of invasive candidiasis.

5.2.4.2. Effect on the cell membrane and cell wall composition

The cell membrane and cell wall of fungi are the most important targets for antifungal drugs. These physical and chemical barriers are responsible for the communication with the environment, for maintaining fungal structure and normal cell growth and, therefore, have a key role in metabolic processes (256, 264). Face to this, the effect of purified phlorotannins extracts on fungal cell wall composition was evaluated by measuring the amount of β-D-(1→3)-glucans and chitin in yeasts and dermatophytes.

5.2.4.2.1. Cell membrane ergosterol

As referred above, ergosterol is responsible for the maintenance of cell membrane structure and functions (156). The effect of purified phlorotannins extracts on fungal membrane composition was evaluated by determining ergosterol by HPLC-DAD, after

fungal treatment with sub-inhibitory extracts concentrations (1/2 to 1/8 of the MIC). With the exception of *C. nodicaulis* and *C. usneoides* that significantly reduced the ergosterol amount in yeast and dermatophyte, respectively, the influence in the cell membrane composition does not seem to be the primary mechanism of phlorotannins antifungal action (**Figure 36**).

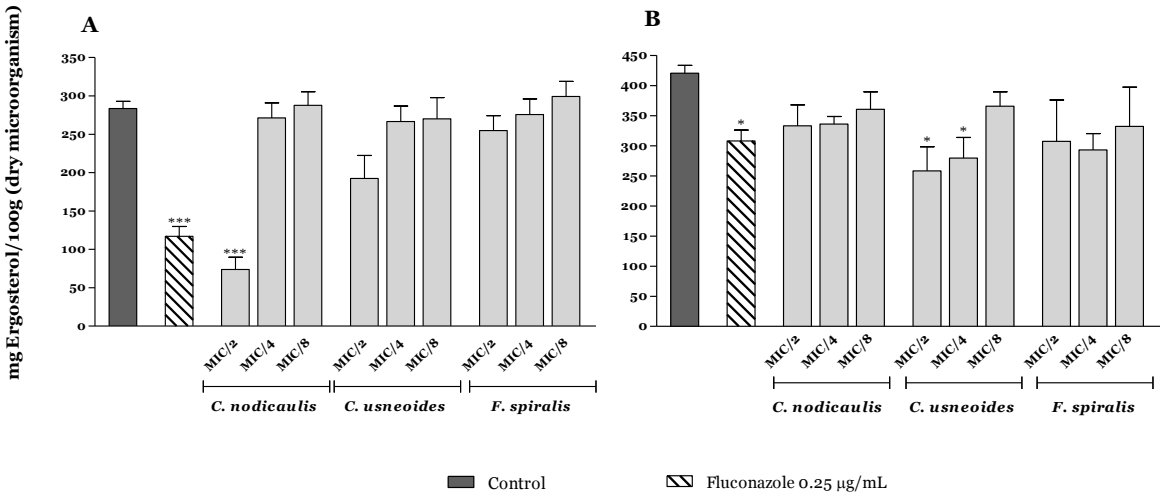

Figure 36. Ergosterol concentration in *C. albicans* ATCC 10231 (A) and *T. rubrum* FF5 (B) cells treated with purified phlorotannins extracts (1/2 to 1/8 of the MIC). Results are expressed as mean (±SD) of three independent assays. **P*<0.05, ***P*<0.01, ****P*<0.001 (319).

For both yeast and dermatophyte, the species *C. nodicaulis* and *C. usneoides,* respectively, reduced the ergosterol amount similarly to fluconazole. This behavior can be related to the seaweeds phlorotannins composition. On one hand, it would be expectable the *Cystoseira* species to act in a similar manner due to genus similarities. On the other hand, regarding the differences related to the phlorotannins amounts and composition of these two species (311, 320), some differences can occur. *C. nodicaulis* has got more phlorotannins than *C. usneoides*; however, *C. usneoides* phlorotannins tend to be of lower molecular weight. Thus, it is possible that phlorotannins with lower molecular weight are more efficient on inhibiting ergosterol biosynthesis in the dermatophyte. Regarding the yeast, it seems that both molecular weight and phlorotannins amount are related to the inhibition of ergosterol biosynthesis. *F. spiralis* is the species with higher phlorotannins amount and, at the same time, the species with higher molecular weight phlorotannins (**Figures 31 and 39, Table 7**). No activity was observed for this species. The phlorotannins molecular weight among *Cystoseira* species is closer and, among them, *C. nodicaulis* presented more activity, having also a higher phlorotannins concentration.

5.2.4.2.2. Cell wall β-D-(1→3)-glucans and chitin

The effect of purified phlorotannins extracts on fungal cell wall composition was evaluated by measuring the amount of β-D-(1→3)-glucans and chitin (data not shown), which are essential components of the fungal cell wall, responsible for maintaining fungal structure and normal cell growth.

β-D-(1→3)-Glucans levels were quantified by a fluorescence assay after microorganisms' treatment with sub-inhibitory concentrations of purified phlorotannins extracts (1/2 to 1/16 of the MIC). Caspofungin, an inhibitor of glucans synthesis, was used as control and the β-D-(1→3)-glucans levels on treated cells were compared with untreated control cells. Chitin levels were determined spectrophotometrically.

Of the studied seaweeds, only *F. spiralis* purified phlorotannins extract significantly reduced the amount of chitin in the dermatophyte *T. rubrum* ($P<0.05$). None of the tested seaweeds extracts affected the β-D-(1→3)-glucans composition of the studied microorganisms. In what concerns to chitin, a much lower amount in yeast than in dermatophyte was expected, as it constitutes only 1-10% of *Candida* cell wall in yeast form (321). However, as this yeast undergoes dimorphic transition in RPMI medium, after the incubation period it is not in the yeast form, rather presents branching filaments, for which chitin composition is similar to that of dermatophytes.

5.2.4.3. Effect on the microorganisms metabolic network

Mitochondria are present in most eukaryotic cells and comprise the respiratory chain. These organelles play several important roles, including generation and regulation of ROS, Ca^{2+} homeostasis, regulation of apoptosis and metabolic processes, being also responsible for more than 90% of cellular ATP production (269). Compounds with the capacity to affect mitochondrial respiratory chain can be seen as potential cell growth inhibitors and capable of trigger cell death (269, 270, 322). With this purpose, the activity of purified phlorotannins extracts was evaluated on the mitochondrial metabolic activity of *C. albicans*. Currently, no antifungal drug primary acts by affecting mitochondria activity.

5.2.4.3.1. Mitochondrial dehydrogenases activity

In order to check whether the phlorotannins could affect the mitochondrial function, the MTT reduction assay was performed with purified phlorotannins extracts concentrations ranging from MIC to MIC/1024 (**Figure 37A**). Surprisingly, *Candida* cells treated with purified phlorotannins extracts presented significantly higher mitochondrial

activity than the control cells, being about 2.5 times higher in cells treated with *C. nodicaulis* extract MIC (**Figure 37 A**). For lower concentrations of extracts, the mitochondrial activity equaled the value of control cells (**Figure 37 A**). Cells were observed under light microscopy and the ones treated with the extracts presented a higher density of formazan salts (**Figure 37 B** and **C**). For *T. rubrum* spores, the MTT conversion rate decreased with increasing phlorotannins concentrations, which was the expected behavior, being in accordance with MIC (data not shown).

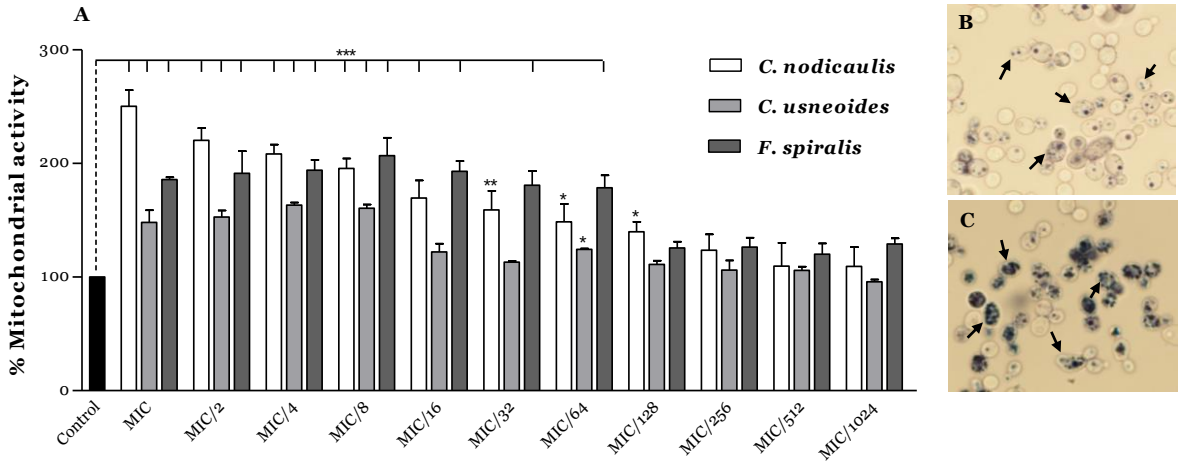

Figure 37. Mitochondrial activity of *C. albicans* ATCC 10231 cells treated with different concentrations of purified phlorotannins extracts. Results are expressed as the percent change of MTT reduction using the non-treated cells as control (mean (±SD) of three independent assays performed in duplicate). For concentrations lower than MIC/1024 the mitochondrial activity was similar to that of the untreated cells. Arrows show the formazan salts produced by *C. albicans* mitochondria. **P*<0.05, ***P*<0.01, ****P*<0.001 (319).

Yeast respiratory chain involves complex and flexible pathways not fully elucidated. *C. albicans* mitochondria contain three respiratory chains: the classical respiratory chain (CRC), a secondary parallel chain (PAR) and an alternative oxidative pathway (AOX). CRC capacity was verified to be twice as large as AOX, while PAR capacity, which was only efficient when both CRC and AOX were blocked, represented only one tenth of the maximal oxygen consumption rate (265, 323).

A large proportion of cellular dehydrogenases, namely succinate, NADH, glycerol 3P-dehydrogenase and LDH, which are part of the CRC, are responsible for the reduction of MTT to formazan salts (318, 323). MTT reduction assay, which is commonly used to assess cell viability by means of the mitochondrial function evaluation, is also used to confirm and determine MIC values. Tetrazolium salts have become some of the most

widely used tools in cell biology for measuring the metabolic activity of cells ranging from mammalian to microbial origin. These salts are cleaved by mitochondrial dehydrogenases to form its purple formazan derivatives, which can be measured and reported to mitochondrial activity (324).

Attending to the results obtained (**Figure 37 A**), and after cells observation under light microscopy (**Figure 37 B** and **37 C**), we can assume a possible stimulation of cellular dehydrogenases, which presented the capacity to convert MTT at a much higher rate than the untreated cells. According to these observations, it can be supposed that the fungistatic activity of phlorotannins on *Candida* cells can be related to toxic effects due to increased ROS production, which follows from the respiratory rate increment (322). During normal respiration, small amounts of toxic intermediate species are generated by partial reduction of O_2, including $O_2^{\bullet-}$, H_2O_2 and $HO^{\bullet}$. Although cells have several enzymatic (superoxide dismutase, GPx and CAT) and non-enzymatic (endogenous and exogenous antioxidants) systems contributing for free radicals inactivation, the reactive species can accumulate and cause cell damage (322, 325). As it is possible that purified phlorotannins extracts increase the mitochondrial respiratory rate by stimulating the activity of yeast dehydrogenases (**Figure 37 A** and **C**), we can assume that these compounds trigger an increased production of ROS. Once the mitochondrial dehydrogenases activity on cells treated with phlorotannins purified extracts was significantly higher ($P<0.001$) than that of control cells, it is possible that the cell's free radical inactivation systems were not able to intercept all of the ROS formed during the respiration process, leading to toxic effects, which inhibit cell division (322).

5.2.4.3.2. Mitochondrial membrane potential

The mitochondrial membrane potential reflects the pumping of hydrogen ions across the inner membrane during the process of electron transport and oxidative phosphorylation, being, together with the mitochondrial dehydrogenases function, a key indicator of cellular viability. The change in fluorescence intensity is related to the inner mitochondrial membrane depolarization, namely to the loss of membrane potential. In this case, mitochondrion loses the ability to sequester Ca^{2+}, resulting in a loss of selectivity of the inner membrane and leading to a mitochondrial membrane permeability transition dependent of Ca^{2+}. This change of permeability is involved in the process of injury and cell death (326, 327). RHO is a fluorescent probe commonly used for the evaluation of mitochondrial membrane potential (293).

The mitochondrial membrane potential was evaluated by measuring the incorporation of the fluorescent probe RHO by *Candida* cells after treatment with purified phlorotannins extracts (**Figure 38**). The amount of RHO incorporated by treated cells was significantly superior to the control for almost all the tested concentrations, increasing from MIC to MIC/8 and decreasing from MIC/16 to MIC/1024 (**Figure 38**). The fluorescence intensity for MIC/1024 equaled the values of untreated cells.

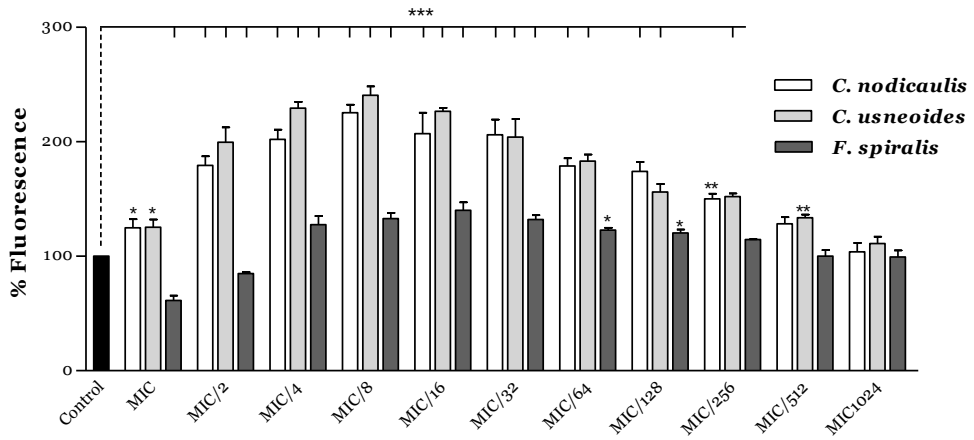

Figure 38. Percentage of Rhodamine 123 fluorescence of *C. albicans* ATCC 10231 cells treated with different concentrations of purified phlorotannins extracts, relative to control. For concentrations lower than MIC/1024 the percentage of fluorescence was similar to that of untreated cells. Results are expressed as mean (±SD) of three independent assays performed in duplicate. **P*<0.05, ***P*<0.01, ****P*<0.001 (319).

Face to the results, it appears that purified phlorotannins extracts have a dual mechanism for regulating the mitochondrial membrane potential (**Figure 38**). The fluorescence intensity was superior to that of untreated cells for almost all extracts concentrations, leading to the assumption that a hyperpolarization state of the mitochondrial membrane could occur (328). Although the increase of fluorescence intensity observed from MIC to MIC/8 was more unexpected, it can be associated with cellular defense mechanisms against apoptosis (329). Taking into account that several cell proteins are responsible for regulating programmed cell death and mitochondria integrity, anti-apoptotic proteins can block cell death by reducing the oxidative stress in cases of severe cellular injury. The expression of these proteins is able to stabilize the mitochondrial membrane potential (329). The mechanism by which membrane polarization occurs is still obscure, although it is known that the loss of mitochondrial membrane potential constitutes an early event during apoptosis in some systems (330-332). As so, a biphasic change in membrane potential can occur for higher concentrations of tested compounds, with an early hyperpolarization followed by depolarization (331). According to this, it could be hypothesized that at the highest concentration (MIC) there is

increased expression of anti-apoptotic proteins by the cell, in a tentative to resist apoptosis and, therefore, a quicker stabilization of the membrane potential takes place. As the concentration of phlorotannins decreases (from MIC to MIC/8), the cellular injury and the expression of anti-apoptotic proteins is reduced, so that the membrane potential is not established as soon.

The second mechanism by which phlorotannins seem to affect the membrane potential is not probably related to anti-apoptotic defenses. The accumulation of ROS inside cells can lead to alterations in cell membranes permeability, leading to the increase of cytosolic Ca^{2+} concentrations, both by the release of Ca^{2+} from the intracellular stores and by the increased influx over the cytoplasmic membrane (322). As soon as the phlorotannins concentration lowers (from MIC/8 to MIC/1024), the membrane potential may be gradually replaced. The cell recovers from the hyperpolarization state, the mitochondrion recovers the membrane potential and the fluorescence value equals the control.

Taking into account such hypotheses, there seems to be two possible mechanisms to explain the behavior of mitochondria in *C. albicans* ATCC 10231 when exposed to phlorotannins. The mechanism taking place for higher phlorotannins concentrations (from MIC to MIC/8) can be regulated by anti-apoptotic proteins expression, once cells are undergoing a major aggression that puts at risk their viability. For lower phlorotannins concentrations (from MIC/16 to MIC/1024) cellular aggression will not be sufficient to trigger anti-apoptotic defense mechanisms and a mechanism related to the change in Ca^{2+} permeability can take place.

The study of the metabolic network of the yeast *C. albicans* revealed a hyperactivity of mitochondrial dehydrogenases caused by phlorotannins. This alteration in mitochondrial activity can lead to the accumulation of ROS and Ca^{2+}, which, in turn, may result in membrane hyperpolarization, incompatible with normal cell metabolism. In order to clarify these observations, the study of the mechanism of antifungal action of phlorotannins needs further investigation, namely in what concerns to their action over some important cell enzymes like proteases and lipases, which play an important role in cell metabolism (333, 334).

This work evidenced the antifungal capacity of phlorotannins against a wide range of yeast and filamentous fungi. In a general way, *C. nodicaulis* revealed to be the most effective species against the studied fungi. Despite not being lethal for *Candida* species under the tested concentrations, this seaweed presented the lowest MIC/MLC for both

yeast and dermatophytes, being also lethal for the last ones. Although the mechanism of action of these compounds was not completely elucidated, there are evidences pointing to some effect on ergosterol and chitin composition in filamentous fungi, and on ergosterol and respiration in yeast. Nevertheless, the main role of phlorotannins seems to be related to their capacity to reduce the virulence factor in *C. albicans*, by inhibiting the dimorphic transition.

The increment in combining antifungal medications with different mechanisms of action can lead to better therapeutic responses. Contrary to what happens with some commercially available antifungal drugs, the effect on yeast mitochondria activity can be the primary mechanism of action of phlorotannins. Thus, the challenge remains to associate compounds from natural matrices with existing antifungal drugs for which there is some resistance. There are some data indicating that phlorotannins do not act similarly in the mitochondria of mammalian and yeast (311). In this sense, searching for differences between mammalian and fungi mitochondria, in the classical and alternative components of the mitochondrial respiratory chain, may provide new potential therapeutic targets in treating pathogenic fungal infections.

Given the high biological potential of phlorotannins and the scarce information concerning the species under study, the evaluation of the biological potential of phlorotannins extracts of the most promising species was further extended. As so, the three *Cystoseira* species and *F. spiralis* were considered for the phlorotannins profiling by LC-MS. The antioxidant potential of these species and their activity on specific enzymes was further studied.

5.2.5. HPLC-DAD-ESI/MS^n phlorotannins analysis

Phlorotannins extracts from the three *Cystoseira* species and *F. spiralis* were chromatographically separated and their mass was analyzed following ESI ionization. The Extracted Ion Chromatogram (EIC) of protonated molecular ions ($[M+H]^+$) from the most common phlorotannins found in literature (dioxinodehydroeckol (MW 371), eckol (MW 373), fucophlorethol (MW 375), 7-phloroeckol (MW 497), fucodiphlorethol (MW 499), phlorofucofuroeckol (MW 603), fucotriphlorethol (MW 623), dieckol (MW 743) and fucophlorethols with six (MW 747), seven (MW 871) and eight units of phloroglucinol (MW 995)) was used for the study of phlorotannins by HPLC-DAD-ESI/MS^n. The MS fragmentations of other peaks observed in the UV chromatogram were studied. The compounds were numbered following elution order, starting by *F. spiralis* (**1**-**8**), and

followed to *C. usneoides* (**9-12**), *C. tamariscifolia* (**13-14**) and *C. nodicaulis* (**15-22**) (**Figure 34**, **Table 6**) (320).

In the EIC of *F. spiralis* and of *C. usneoides* it could be noticed the presence of well-defined and abundant ions, **1-8** and **9-12**, respectively, which can correspond to phlorotannins. On the other hand, in the EIC of *C. tamariscifolia* and *C. nodicaulis*, the ions were found in trace amounts and appear co-eluting with other compounds (**Figure 39**) (320).

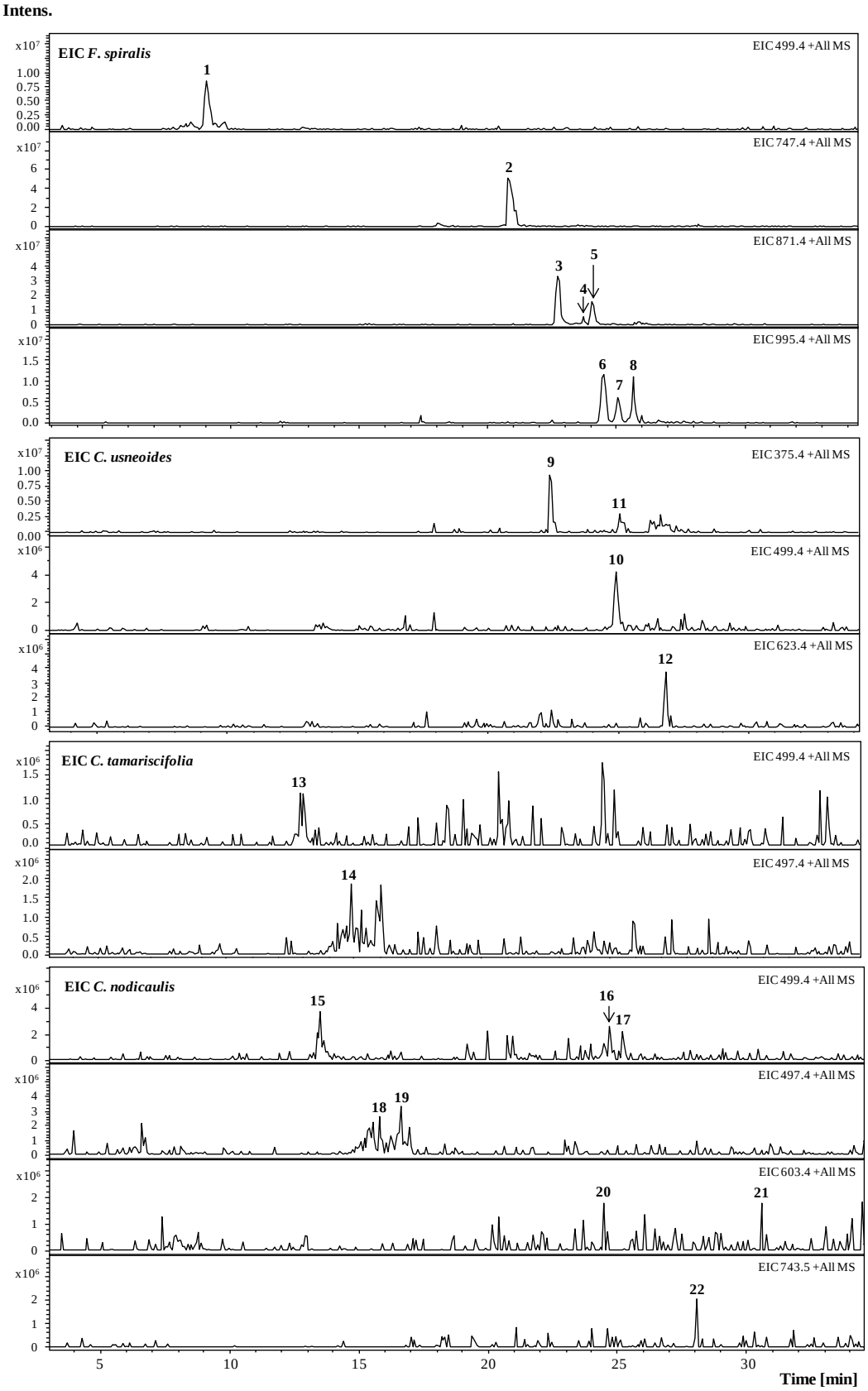

Figure 39. Extracted Ion Chromatogram (EIC) of purified phlorotannins extracts of *F. spiralis*, *C. usneoides*, *C. tamariscifolia* and *C. nodicaulis*. $[M + H]^+$ (*m/z*): 1, 499; 2, 747; 3-5, 871; 6-8, 995; 9 and 11, 375; 10, 499; 12, 623; 13, 499; 14, 497; 15-17, 499; 18-19, 497; 20-21, 603; 22, 743 (320).

Table 7. *R*t, UV and MS: $[M+H]^+$, $+MS^2[M+H]^+$ data of compounds from *F. spiralis* (**1-8**), *C. usneoides* (**9-12**), *C. tamariscifolia* (13-14) and *C. nodicaulis* (**15-22**)[a] (320).

	***R*t**	**UV (nm)**	**$[M + H]^+$,**	**$+MS^2$ $[M + H]^+$, *m/z*(%)**
1	9.0	---	499	481(80, -18), 439 (53, -18-42), 392 (25), 373 (35, -126), 355 (100, -126-18)
2	20.8	248, 268sh	747	729(45, -18), 711 (70, -36), 585 (25, -126-36), 571 (35, -126-36-14), 220 (100)
3	22.7	244, 270sh	871	853(100, -18), 835 (60, -36), 727 (30, -126-18), 709 (55, -126-36), 681 (30), 641 (12), 585 (15), 570 (7), 484 (35)
4	23.6	---	871	853(100, -18), 835 (76, -36), 727 (38, -126-18), 709 (17, -126-36), 603 (22), 585 (20), 517 (9), 403 (7), 310 (4)
5	24.0	274sh	871	853(67, -18), 835 (100, -36), 725 (25), 709 (55, -126-36), 694 (58), 681 (21), 569 (17), 551 (18), 435 (20)
6	24.4	272sh	995	977(100, -18), 959 (45, -36), 851 (3, -126-18), 833 (15, -126-36), 709 (26, -126-124-36), 692 (15)
7	25.0	274sh	995	977(100, -18), 959 (27, -36), 869 (7, -126), 851 (8, -126-18), 833 (12, -126-36), 709 (9, -126-124-36), 692 (6)
8	25.6	274sh	995	977(100, -18), 959 (8, -36), 851 (19, -126-18), 833 (14, -126-36), 709 (12, -126-124-36), 692 (14)
9	22.5	270	375	357(44, -18), 339 (2, -36), 249 (42, -126), 235 (100, -126-14), 231 (74, -126-18), 217 (43), 127 (75, floroglc + H)
10	25.0	---	499	481(80, -18), 453 (18, -18-28), 419 (70), 372 (33, -127), 359 (30, -126-14), 355 (100, -126-18), 301 (42), 179 (43)
11	25.2	---	375	357(23, -18), 339 (7, -36), 249 (40, -126), 235 (100, -126-14), 317 (33), 127 (57, floroglc + H)
12	26.8	---	623	605(33, -18), 577 (18, -18-28), 523 (50), 497 (2, -126), 483 (12, -126-14), 479 (10, -126-18), 337 (44), 231 (100)
13	13.0	---	499	481(49, -18), 411 (29), 397 (28), 395 (25), 359 (25, -162-14), 356 (17), 250 (16), 231 (100), 166 (33)
14	14.9	---	497	479(9, -18), 462 (24), 451 (30, -18-28), 386 (48), 368 (70), 351 (34), 258 (100), 240 (38), 222 (37)
15	13.5	---	499	481(70, -18), 373 (24, -126), 355 (70, -126-18), 341 (52, -126-18-14), 291 (33), 272 (70), 249 (100)
16	15.8	---	497	479(100, -18), 463 (24), 451 (31, -18-28), 368 (8), 351 (45), 298 (14), 258 (41)
17	16.7	---	497	479(83, -18), 462 (100), 452 (8), 435 (18), 385 (32), 368 (74), 351 (23), 258 53)
18	24.5	---	603	559(100, -44), 482 (94), 464 (78)
19	24.7	---	499	481(100, -18), 368 (38), 247 (25), 242 (25)
20	25.2	---	499	462(9), 368(100), 334(12),
21	28.1	---	743	725(45, -18), 715 (80, -28), 701 (97, -42), 685 (10), 633 (100), 600 (33)
22	30.5	---	603	585(100, -18), 523 (15), 458 (17), 368 (56), 301 (30)

[a] Main observed fragments. Other ions were found but they have not been included.

The UV chromatograms of the four studied species extracts were recorded at 280 nm and exhibited some abundant peaks that do not correspond to any of the studied ions (**Figure 40**). Ions corresponding to the compounds tentatively identified as phlorotannins are not abundant in the chromatograms of *C. tamariscifolia* and *C. nodicaulis*.

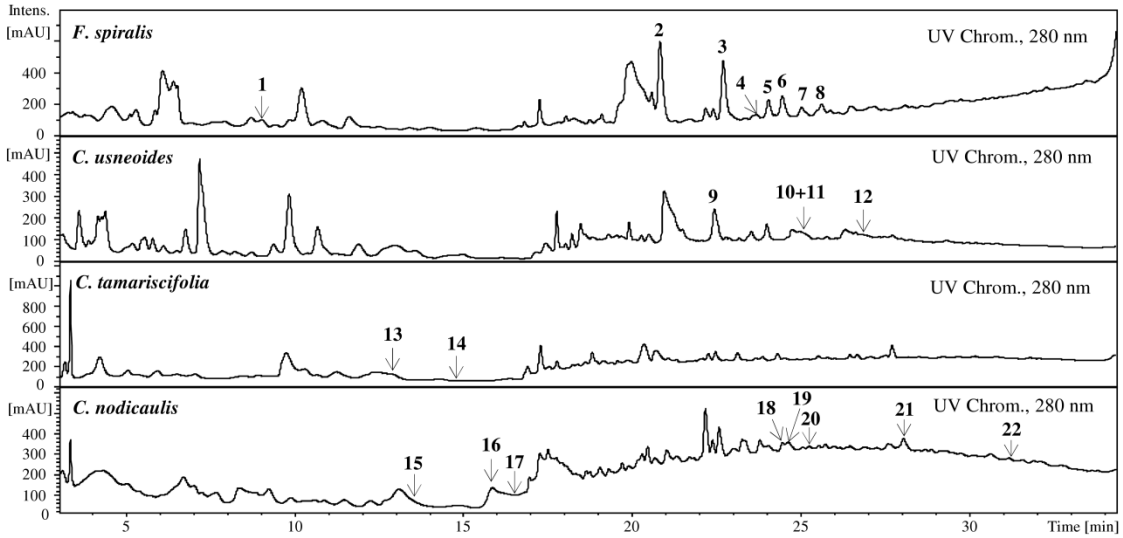

Figure 40. UV chromatograms of the purified phlorotannins extracts of *F. spiralis*, *C. usneoides*, *C. tamariscifolia* and *C. nodicaulis*, recorded at 280 nm. Identity of compounds as in **Figure 39** (320).

The MS study of the ions of possible phlorotannins allowed the detection of two isomers, **9** and **11**, in *C. usneoides*, which protonated molecular ions ($[M+H]^+$) at *m/z* 375 correspond to fucophlorethol isomers (**Figure 41**).

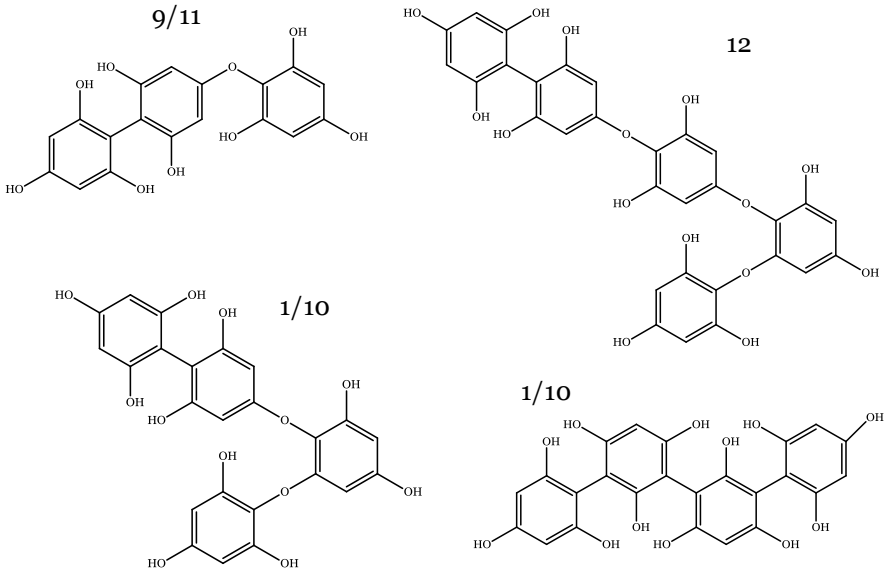

Figure 41. Hypothetical structure of the identified phlorotannins: identity of compounds as in **Figure 39**.

These compounds present a similar fragmentation, with losses of 1 and 2 molecules of H_2O (-18 and -36, respectively), phloroglucinol (-126), as well as the protonated molecular ion of phloroglucinol (*m/z* 127), base peak being the ion resulting from the loss of phloroglucinol and methyl (*m/z* 235, -126-14) (**Figure 42 B**, **Table 7**). In fact, isomers of fucophlorethols with the same molecular ion can occur, depending on the position of the aryl linkage (141). There were also observed isomers with $[M+H]^+$ at *m/z* 499, phlorotannins tetramers, compounds **1** in *F. spiralis* and **10** in *C. usneoides*, which can correspond to tetrafucol and/or fucodiphlorethol (**Figure 41**). Their MS^2 shows losses similar to those listed above (-18, -126/-127, among others) and the base peak in both is the ion at *m/z* 355 (-126-18). These losses were also detected in the other compounds, which protonated molecular ions indicated them as polymers with several phloroglucinol units. Thus, in *C. usneoides* compound **12** (with protonated molecular ion at *m/z* 623) was suggested to be a polyphenolic compound composed by five phloroglucinol units, possibly a fucotriphlorethol (**Figure 41**). In *F. spiralis* compound **2** ($[M+H]^+$ at *m/z* 747) can be a polyphenolic compound composed of six units of phloroglucinol. Three isomers (compounds **3-5**) with *m/z* 871, corresponding to seven phloroglucinol units, and other three (compounds **6-8**) with *m/z* 995 (eight units of phloroglucinol) were also detected in this species (**Figure 42 A, Table 7**) (320).

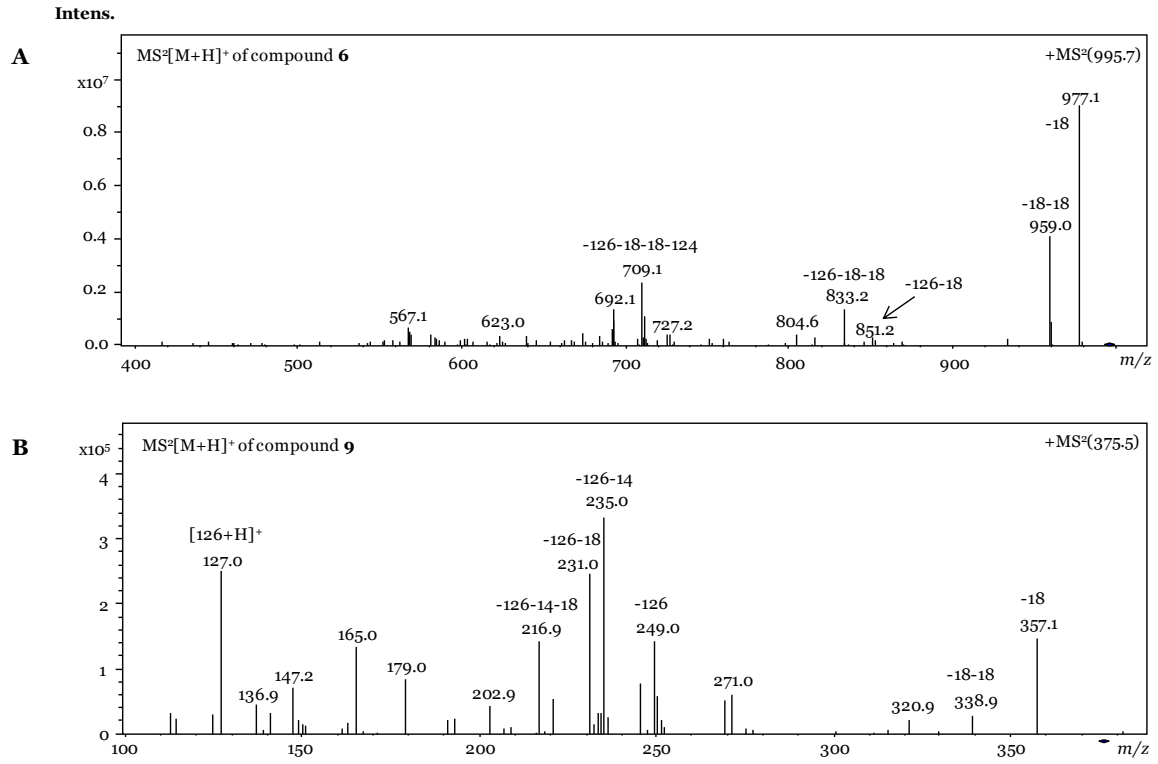

Figure 42. $MS^2[M+H]^+$ analysis of compound 6 from *F. spiralis* (**A**) and compound 9 from *C. usneoides* (**B**) (320).

Species belonging to the order Fucales (119, 211, 314, 322) and Laminariales (103, 335) have been the subject of many studies, allowing the identification of several phlorotannins structures, such as eckol, dieckol, 8,8' dieckol, 6,6' bieckol, 8,8' bieckol, phlorofucofuroeckol-A and B, fucofuroeckol-A, dioxinodehydroeckol, 2-phloroeckol, 7-phloroeckol, fucodiphlorethol-G and triphlorethol. Thus, in the present work 22 different phlorotannins belonging to eckol and fucophlorethol main groups were characterized in the studied seaweeds: 8 in *C. nodicaulis*, 2 in *C. tamariscifolia*, 4 in *C. usneoides* and 8 in *F. spiralis*) (**Figures 39** and **40, Table 7**) (320).

More recently, Tierney and co-workers profiled *F. spiralis* phlorotannins by UPLC-MS, starting from cold H_2O and ethanol:H_2O extracts (336). On the cold H_2O extract those authors detected ions corresponding to 498, 746, 870 and 994 g/mol. In the ethanol:H_2O extract, ions with 746, 870 and 994 g/mol were observed. Those authors also identified phlorotannins with a higher degree of polymerization that may be explained by the use of UPLC, which allows an increased efficiency in separation than HPLC. Our results are in agreement with those authors in what concerns to the molecular weight of phlorotannins of the cold H_2O extract. Nevertheless, and even highlighting the degree of polymerization of phlorotannins, those authors have not made an approach to the chemical phlorotannin group. Anyway, the complexity of these molecules remains a challenge in terms of chromatographic separation and chemical characterization. Thus, with the exception of fucols and fucophlorethols groups identified in *F.* spiralis (316), and bifuhalol and diphlorethol identified in *C. tamariscifolia* (322), none of the compounds described herein was previously reported in these species.

Concerning the mode of action of phlorotannins, there is also a recent survey conducted by Hierholtzer and co-workers, where a crude phlorotannins extract with known concentration was tested against mixed anaerobic microbial cultures (337). The authors concluded that the bactericidal activity of phlorotannins was function of the level of polymerization of the compounds present in the extract. They also observed that microorganisms presented a higher energy consumption for survival, which resulted from the stress induced by phlorotannins (337).

5.2.6. Antioxidant Activity

The antioxidant potential of purified phlorotannins extracts from the three *Cystoseira* species and *F. spiralis* was checked against $O_2^{-\cdot}$ and LPO. A concentration-dependent pattern was observed for both assays. *C. nodicaulis* and *F. spiralis* were the species with the highest radical scavenging capacity, presenting IC_{50} values of 0.93 and 1.30 mg/mL (dry weight), respectively (**Figure 43 A, Table 8**). The IC_{50} found for these

two species was significantly lower than those obtained for *C. tamariscifolia* and *C. usneoides* ($P<0.0001$). Concerning LPO inhibition, *F. spiralis* was the most effective, displaying an IC_{50} value (2.32 mg/mL, dry weight) significantly lower than those found for both *C. nodicaulis* and *C. tamariscifolia* ($P<0.0001$) (**Figure 43 B, Table 8**). The protective activity of *C. usneoides* against LPO was not higher than 15% for the highest concentration tested (9.1 mg/mL dry weight, **Table 8**).

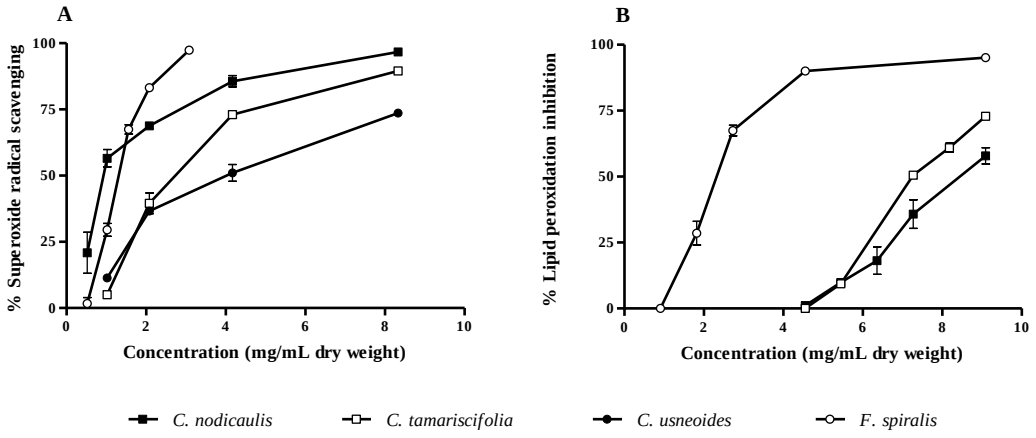

Figure 43. Activity of purified phlorotannins extracts against superoxide radical (**A**) and lipid peroxidation (**B**). Results are expressed as percentage relative to control (mean ± standard deviation of three independent assays) (320).

Table 8. Antioxidant activity and HAase inhibition of purified phlorotannins extracts [a] (320).

	Assays		
Samples	**Superoxide radical scavenging**	**Lipid peroxidation inhibition**	**HAase inhibition**
C. nodicaulis	0.93 (0.11)	8.52 (0.08)	1.34 (0.04)
C. tamariscifolia	2.73 (0.30)	7.55 (0.02)	3.3 (0.12)
C. usneoides	4.02 (0.68)	> 9.10	2.51 (0.22)
F. spiralis	1.30 (0.04)	2.32 (0.08)	0.73 (0.06)

[a] IC_{50} values (mg/mL, dry weight) represented as mean (standard deviation) of three assays, performed in triplicate.

The peroxidation of lipids is one of the inevitable causes of oxidative stress, because the derived products lead to the spread of free radical reactions (222, 338). As so, the anti-radical activity is of extreme importance to establish a balance between pro-oxidants/antioxidants. The role of antioxidants takes a crucial importance when the subject is the skin. This physical and chemical barrier is subjected to a wide kind of aggressions during life, which are reflected in the loss of its integrity and properties, resulting in diverse situations, among which aging can be highlighted. Skin aging has been associated with the increased peroxidation of skin lipids, namely of squalene, which constitutes the main component of skin surface polyunsaturated lipids and is easily peroxided. Peroxidation of fatty acyl groups of membrane phospholipids is one of the major outcomes of free radical-mediated injury to tissue, leading to cell damage by continuous chain reactions. In addition, LPO may be responsible for the activation of phospholipase A2, production of many mediators by arachidonate, deactivation of adenylate cyclase and activation of guanilate cyclase, leading to the decrease of cAMP/cGMP ratio, responsible for epidermal proliferation. Thus, the physicochemical properties of the membrane lipid bilayers can be greatly altered, resulting in severe cellular dysfunction (338).

Several works have concerned the antioxidant activity of phlorotannins crude extracts and isolated compounds. Zubia and co-workers assessed the antioxidant capacity of 10 brown seaweeds from the Brittany coast (339). Species belonging to the order Fucales, mainly those of the genus *Fucus* and *Cystoseira*, displayed the strongest activity, which was positively correlated with their phenolics content. Similar results were obtained by Shibata and colleagues with phlorotannins isolated from Laminariaceous (103). These authors found that the increase of molecular weight of the isolated phlorotannins leads to a decrease of the antioxidant activity. According to these previous reports, it is possible to establish a correlation between phlorotannins composition and biological activity. Thus, *F. spiralis* is the species displaying the lowest IC_{50} regarding LPO inhibition and *C. nodicaulis* the one showing best superoxide scavenging capacity, but almost no activity against LPO (**Figure 43, Table 8**). It seems that extracts with lower molecular weight phlorotannins are more effective as superoxide radical scavengers, while those with higher molecular weight are better in inhibiting LPO (**Tables 7** and **8**). Anyway, the mode of action of these molecules and the phlorotannins groups responsible for each activity are not easy to point, moreover dealing with an extract instead of isolated compounds. Thus, synergistic and antagonic effects can be behind the main molecules and affect the results. Recent studies have demonstrated that the protective effect against oxidative stress induced by ROS and UV radiation was connected to the number and position of hydrogen-

donating OH groups on the aromatic ring of the phenolic molecules, being also affected by other factors, such as other hydrogen donating groups (-NH, -SH) (340). A recent review about cosmeceuticals derived from marine seaweeds highlighted the importance of marine polyphenols, particularly phlorotannins, because of their antioxidant activity, stronger than that of many other polyphenols, which may be related, in part, to their molecular arrangement (341).

Kang and co-workers have recently studied the effect of the pre-treatment with dieckol on the LPO observed in mice liver. These authors concluded that dieckol, a low molecular weight phlorotannin, dramatically reduces LPO induced by ethanol (342). With the same purpose, another research group (197) proved that phlorotannins can reduce the LPO induced by UV radiation. Since skin is always exposed to UV radiation and environmental pollutants, it is a main target of oxidative stress. The overexposure of skin to UV radiation is associated with abnormal cutaneous reactions, such as epidermal hyperplasia, accelerated breakdown of collagen and inflammatory responses. The effect of UV radiation has a strong oxidative component and photo-oxidative stress has been directly linked to the onset of skin photodamage. Thus, regular skin treatments with products containing antioxidant ingredients can be useful for the prevention and reduction of damage induced by UV radiation (343, 344).

As it has been seen before, despite their capacity to inhibit LPO and their antioxidant activity against $O_2^{\bullet-}$, *F. spiralis* and *C. nodicaulis* phlorotannins extracts have demonstrated the highest capacity for NO scavenging. Since NO reacts rapidly with superoxide radical to form peroxynitrite ($ONOO^-$), the studied species can prevent the generation of $ONOO^-$, a strong oxidant with a lethal effects on many cells (280).

5.2.7. Inhibitory Effect on HAase

HAase, an enzyme responsible for the depolymerization of HA (a polysaccharide present in the extracellular matrix of the connective tissue and in body fluids), is known to be involved in allergic effects, migration of cancer and inflammation, increasing the permeability of the vascular system. As so, potent HAase inhibitors might have anti-allergic, anticancer and anti-aging activities, becoming leading compounds in the development of new drugs (223, 317, 345). Of the species studied herein, *F. spiralis* demonstrated the highest capacity to inhibit HAase (IC_{50}=0.73 mg/mL dry weight), *C. tamariscifolia* being the less effective (**Figure 44**, **Table 8**). All of the IC_{50} values found were significantly different (P<0.0001).

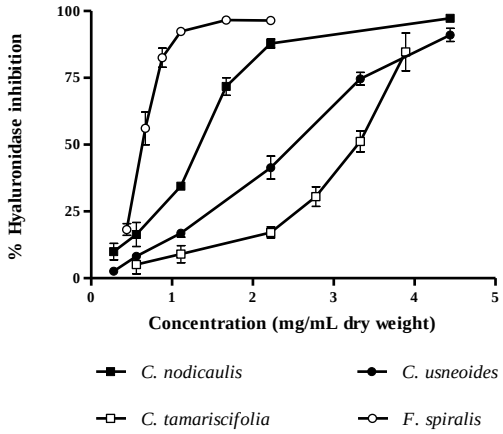

Figure 44. Inhibitory activity of purified phlorotannins extracts on hyaluronidase. Results are expressed as percentage of hyaluronidase inhibition relative to control (mean ± standard deviation of three independent assays) (320).

Phlorotannins are reported as anti-allergic and potential anti-aging agents, owing to their strong inhibitory effect on HAase. Sugiura and co-workers performed a bioguided fractionation of a MeOH:chloroform extract of the brown seaweed *Eisenia arborea* Areschoug and identified six phlorotannins, phlorofucofuroeckol-B showing the strongest activity (335). HAase inhibition assay was also performed with crude phlorotannins extracted from five brown seaweeds from Pakistan and China (346). A positive correlation between the extracts activity and the total phlorotannins content was observed. *Sargassum tennerimum* J. Agardh was the most active species, exhibiting an IC_{50} value lower than that of disodium cromoglycate, a natural inhibitor of HAase. In addition, Shibata and colleagues observed that phlorotannins like eckol, phlorofucofuroeckol A, dieckol, and 8,8'-bieckol exert a stronger inhibition of HAase than well-known inhibitors, such as catechin and sodium cromoglycate, and that phlorotannins with higher molecular weight are more effective (110). The inhibition on HAase is more than one strategy to reduce the progression of allergy and inflammation. The inhibition of this enzyme is the first step for the preservation of the HA structure and, thus, the maintenance of the skin framework. Thus, the activity of phlorotannins can be further explored concerning not only inflammatory and allergic diseases, but also skin aging which, more than fashionable, is a matter of self-esteem.

Our findings seem to be in good agreement with previous reports. In fact, the best results obtained with *F. spiralis* extract are not surprising, as this species possesses the highest total phlorotannins content (311). Furthermore, it also contains the ones with higher molecular weight, as demonstrated herein (**Figure 39, Table 7**). However, the

structure of phlorotannins appears to be more important than its amount. The genus *Cystoseira* is a good example: despite having a much lower phlorotannins content than *C. tamariscifolia* (311), *C. nodicaulis* and *C. usneoides* presented significantly lower IC_{50} values for HAase inhibition (**Figure 44**, **Table 8**).

5.3. Further exploration of *F. spiralis* bioactive compounds

The recognition of *F. spiralis* as nutritionally and pharmacologically promising, and considering its high content of bioactive compounds, stimulated us to go further in terms of compounds' isolation and determination of their biological activities. With this purpose, the lyophilized seaweed material was exhaustively extracted with MeOH and the resulting MeOH extract was subjected to solvent-solvent partitioning to give n-hexane, EtOAc and BuOH fractions. The HPLC-UV monitorization of the compounds present in each solvent revealed the EtOAc as the richest one, as its chromatogram presented a higher amount and intensity of peaks. Thus, EtOAc fraction was chosen for further analysis.

5.3.1. Isolation of monoacylglycerol and MGDGs

The EtOAc fraction was partitioned as outlined in the experimental section to yield one monoacylglycerol featuring oleic acid (C18:1 Ω9) (compound **1**) and a 1:1 mixture of two MGDGs containing EPA (C20:5 Ω3) combined with octadecatetraenoic acid (C18:4 Ω3) (compound **2**) and LNA (C18:3 Ω3) (compound **3**), respectively (**Figure 45**). Their structures and the regiochemical attachments of the acyl chains to the glycerol moiety were unambiguously elucidated on the basis of extensive NMR spectroscopic analysis and MS and by comparison with data reported in the literature (217, 347, 348).

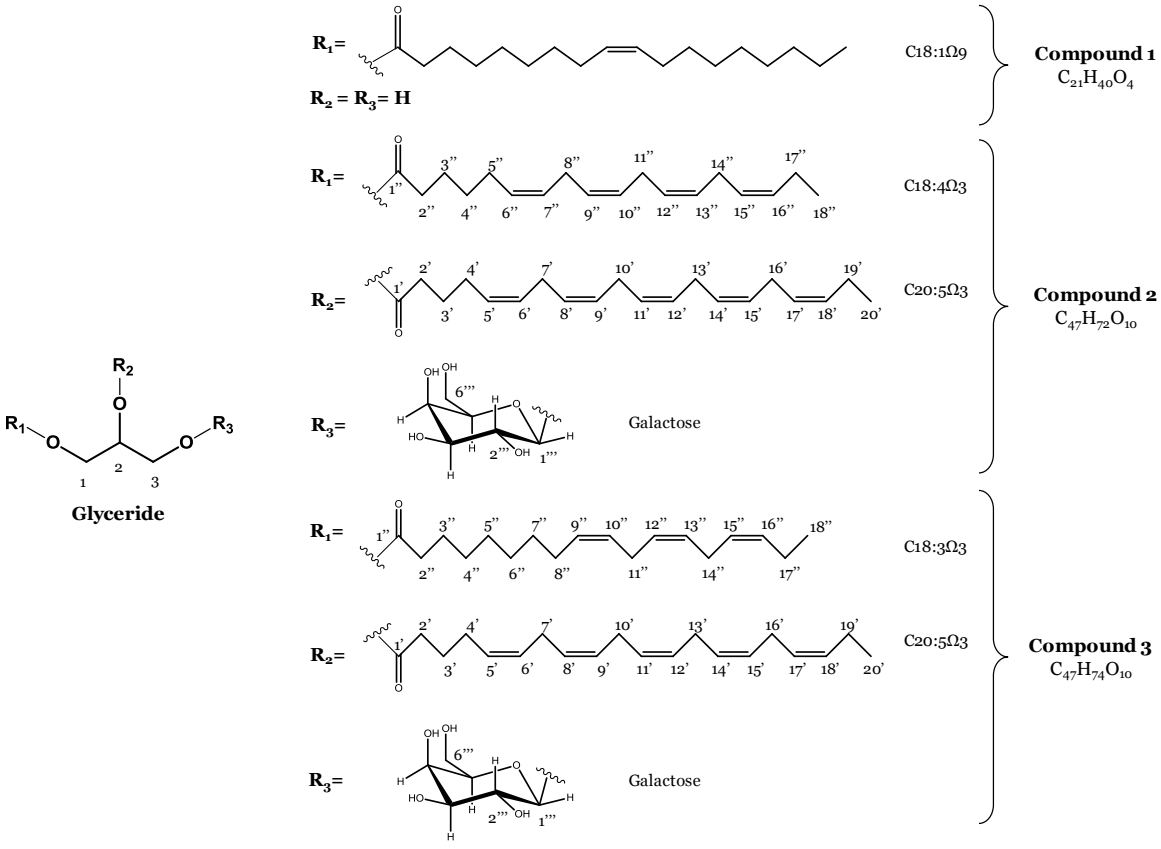

Figure 45. Structures of the monoacylglycerol (compound 1) and the MGDGs (compounds 2 and 3) isolated from *F.spiralis*.

Previous reports also include the isolation of compounds 2 and 3 from the fresh water dinoflagellate *Glenodinium sanguineum* Marchesoni (349) and their detection in glaucocystophytes (350).

5.3.2. Spectrometric data of isolated compounds

The spectrometric data of the monoacylglycerol (compound 1 – **Figure 45**) were previously reported by Hirao and co-workers in the brown seaweed *Ishige sinicola* (Setchell & N. L. Gardner) Chihara (347) and are in agreement with the data obtained in this work with *F. spiralis*, in which this compound was reported for the first time:

Compound **1** (**Figure 45**): ^{1}H NMR (500 Mz, CDCl3): 5.35 (2H, m), 4.19 (1H, dd, *J* = 11.7, 4.5 Hz), 4.13 (1H, dd, *J* = 11.7, 6.0 Hz), 3.91 (1H, m), 3.68 (1H, dd, *J* = 11.7, 4.0 Hz), 3.58 (1H, dd, *J* = 11.7, 6.0 Hz), 2.33 (2H, t, *J* = 7.6 Hz), 2.01 (4H, m), 1.61 (2H, m), 1.29 (22H, m), 0.86 (3H, t, *J* = 6.9 Hz); ESIMS positive *m/z* 357 $[M+H]^+$ (100), 735 $[2M+Na]^+$ (5) (**Figure 46**); in accordance with literature (347).

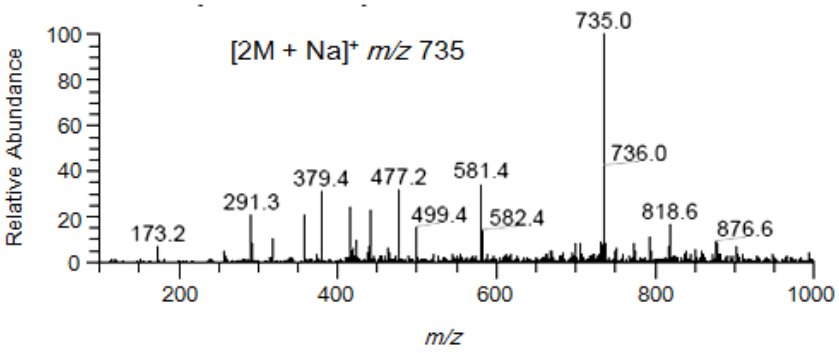

Figure 46. Positive mode ESI - Full mass spectrum [100.00-1000.00] of compound 1.

Data related to the MGDGs was also previously reported in the brown seaweed *Sargassum thunbergii* (Mertens ex Roth) Kuntze (188) and later in the diatom *Phaeodactylum tricornutum* Bohlin (348):

Compounds **2** and **3** (**Figure 45**): ^{1}H NMR (CDCl3, 500 Mz) of glycerol and sugar parts, common to both compounds: 4.38 (1H, dd, 12.0, 3.3, H-1a), 4.19 (1H, dd, 12.0, 6.4, H-1b), 5.30 (1H, m, H-2), 3.89 (1H, dd, 11.0, 5.6, H-3a), 3.73 (1H, dd, 11.0, 6.4, H-3b), 4.26 (1H, d, 7.4, H-1'''), 3.63,(1H, dd, 9.5, 7.4, H-2'''), 3.58 (1H, dd, 9.5, 2.5, H-3'''), 3.99 (1H, brd, 2.5, H-4'''), 3.53 (1H, brt, 4.6, H-5'''), 3.96 (1H, dd, 12.5, 5.8, H-6a'''), 3.85 (1H, dd, 12.5, 4.6, H-6b'''); ^{13}C NMR (CDCl3, 500 Mz): 63.1 (C-1), 70.4 (C-2), 68.5 (C-3), 104.2 (C-1'''), 71.8 (C-2'''), 73.6 (C-3'''), 69.5 (C-4'''), 74.7 (C-5'''), 62.7 (C-6'''); in accordance with literature (188, 348).

Compound **2** (**Figure 45**): ^{1}H NMR (CDCl3, 500 Mz): 5.36 (18H, m, H-5', 6', 8', 9', 11', 12', 14', 15', 17', 18', 6'', 7'', 9'', 10'', 12'', 13'', 15'', 16''), 2.80 (14H, m, HC-7', 10', 13', 16', 8'', 11'', 14''), 2.31 (4H, m, H-2', 2''), 2.08 (2H, m, H-4'), 2.05 (6H, m, H-5'', 17'', 19'), 1.67 (2H, m, H-3'), 1.61 (2H, m, H-3''), 1.37 (2H, m, H-4''), 0.96 (6H, t, J = 7.5, H-18'', 20') and ^{13}C NMR (CDCl3, 500 Mz): 174.1 (C-1'), 173.5 (C-1''), 33.7 (C-2'), 34.4 (C-2''), 25.1 (C-3', 3''), 26.7 (C-4'), 29.4 (C-4''), 129.2 (C-5'), 27.1 (C-5''), 128.1-129.0 (C-6', 8', 9', 11', 12', 14', 15', 7'', 9'', 10'', 12'', 13''), 129.8 (C-6''), 25.8 (C-7', 10', 13', 16', 8'', 11'', 14''), 127.2 (C-17', 15''), 132.2 (C-18', 16''), 20.7 (C-19', 17''), 14.3 (C-20', 18''); ESIMS positive *m/z* 819 $[M+Na]^+$ (10), 797 $[M+H]^+$ (100), 635 $[M+H-C_6H_{10}O_5]^+$ (24), 617 $[M+H-C_6H_{12}O_6]^+$ (1), 333 $[C_{17}H_{28}CO + 74]^+$ (3), 285 $[C_{19}H_{29}CO]^+$ (0.5) (**Figure 47**); in accordance with literature (188, 348).

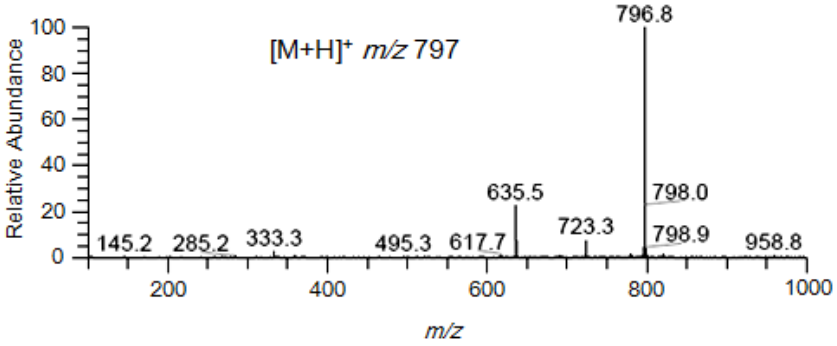

Figure 47. Positive mode ESI - Full mass spectrum [100.00-1000.00] of compound 2.

Compound **3** (**Figure 45**): ^{1}H NMR (CDCl3, 500 Mz): 5.36 (16H, m, H-5', 6', 8', 9', 11', 12', 14', 15', 17', 18', 9", 10", 12", 13", 15", 16"), 2.80 (12H, m, H-7', 10', 13', 16', 11", 14"), 2.31 (4H, m, H-2', 2"), 2.08 (2H, m, H-4'), 2.05 (4H, m, H-19',17"), 2.04 (2H, m, H-8"), 1.67 (2H, m, H-3'), 1.59 (2H, m, H-3"), 1.31 (8H, m, H-4", 5", 6", 7"), 0.96 (6H, t, *J* = 7.5, H-18", 20"); ^{13}C NMR (CDCl3, 500 Mz): 173.8 (C-1'), 173.5 (C-1"), 33.7 (C-2'), 34.4 (C-2"), 25.1 (C-3'), 24.9 (C-3"), 26.7 (C-4'), 29.3 (C-4"), 129.2 (C-5'), 29.5 (C-5"), 128.1-129.0 (C-6', 8', 9', 11', 12', 14', 15', 10", 12", 13"), 29.8 (C-6"), 25.8 (C-7', 10', 13', 16', 11", 14"), 29.9 (C-7"), 27.4 (C-8"), 130.2 (C-9"), 127.2 (C-17', 15"), 132.2 (C-18', 16"), 20.7 (C-19', 17"), 14.3 (C-20', 18"); ESIMS positive *m/z* 821 $[M+Na]^+$ (9), 799 $[M+H]^+$ (100), 637 $[M+H-C_6H_{10}O_5]^+$ (23), 619 $[M+H-C_6H_{12}O_6]^+$ (1), 335 $[C_{17}H_{30}CO + 74]^+$ (2), 285 $[C_{19}H_{29}CO]^+$ (0.6) (**Figure 48**); in accordance with literature (188, 348).

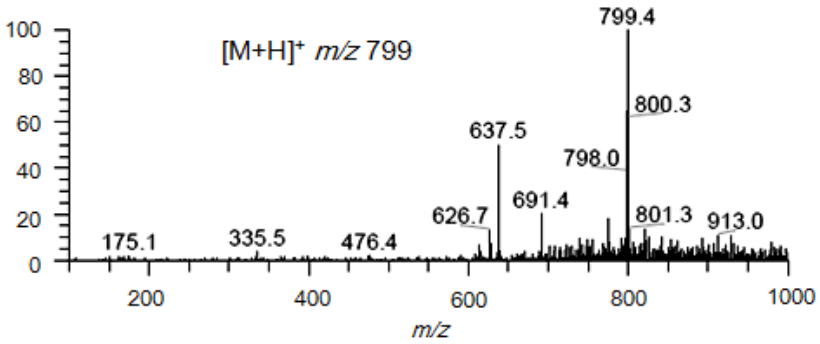

Figure 48. Positive mode ESI - Full mass spectrum [100.00-1000.00] of compound 3.

5.3.3. Anti-inflammatory activity

The anti-inflammatory activity of this kind of compounds has been studied in diferent cell lines and more extensively on cells from articular cartilage (351, 352). Nevertheless, to our knowledge, there were no reports concerning the anti-inflammatory

activity of the glycerolipids isolated from *F. spiralis*. Thus, the anti-inflammatory capacity of the isolated compounds was assessed in the macrophage cell line RAW 264.7.

5.3.3.1. Cell viability

The viability of RAW 264.7 macrophages was evaluated using non-treated cells, in order to assess the effect of 18 h of exposure to 1 μg/mL LPS. Cell viability, as evaluated by the LDH and MTT assays, was not significantly different from that of non-exposed control cells (**Figure 49**).

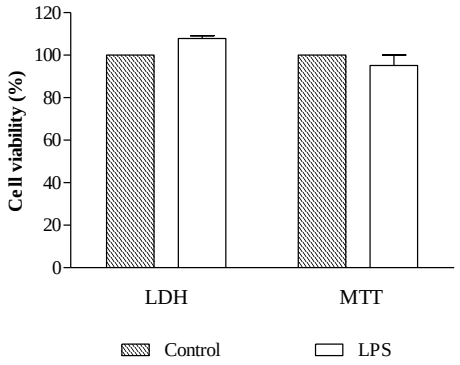

Figure 49. Influence of LPS in cell viability. RAW 264.7 macrophages were exposed to 1 μg/mL of LPS during 18 h and cell viability was assessed by LDH and MTT assays. Results are expressed in percentage of control (mean ± SEM of four independent assays performed in duplicate).

The cytotoxicity of the monoacylglycerol (compound **1**) and of the fraction containing MGDGs (compounds **2** and **3**), in a ratio of 1:1, isolated from *F. spiralis* was also evaluated by the MTT and LDH assays, prior to testing them for their anti-inflammatory activity, following the same procedure as for purified phlorotannins extracts (**Figure 50**).

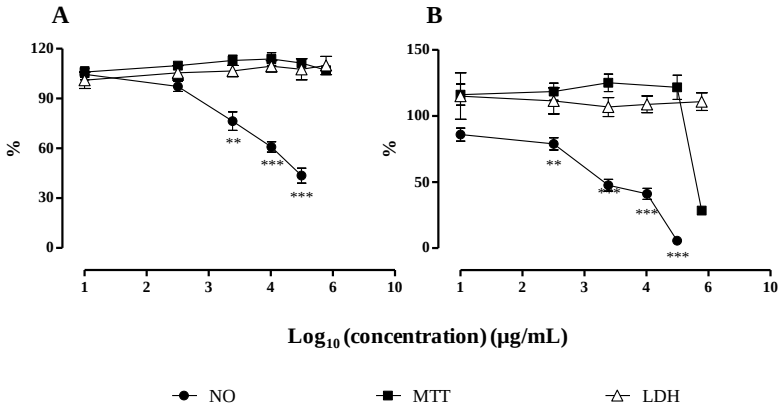

Figure 50. Influence of the monoacylglycerol (A) and of MGDGs (B) on cell viability and on NO released by macrophages. After pre-exposure with the tested compounds and stimulation with LPS, the viability of RAW 264.7 cells was assessed by the LDH and MTT assays and the NO production was quantified. Results are expressed as percentage of control (mean ±SEM of four independent assays performed in duplicate). ***P*<0.01, ****P*<0.001.

Concerning the monoacylglycerol, exposure to LPS had no effect on cell viability at the tested concentrations (6.25 – 100 μg/mL). There was no statistical difference relative to control for both MTT and LDH assays (**Figure 50A**) and the microscopic evaluation revealed normal size and shape of the cells. The same pattern was observed in control cells, treated with the monoacylglycerol but without LPS (data not shown).

On the other hand, cells treated with the highest concentration of the MGDGs fraction (500 μg/mL) suffered a decrease of cell viability to 33.06 ± 3.68%, relative to control, in MTT assay (P<0.001) (**Figure 50B**). This assay, which provides information about the mitochondrial function of the cells by evaluating succinate dehydrogenase activity, proves that there was a drastic loss of viability (353). Curiously, this effect was not confirmed with the LDH assay, which presented values around 100% relative to control for all the tested concentrations (**Figure 50B**). As so, the lack of LDH in the extracellular medium indicates that there was no cell membrane damage.

In order to confirm these results, cells were observed under light microscopy, immediately before starting with the treatment (this is, before pre-exposing the cells for 1 h to the tested compounds) and after the established incubation period (1 h exposure to the tested compounds followed by 18 h incubation with both compounds and LPS). This allowed us to observe that before exposure to test compounds and 1 h after exposure, macrophages presented normal size and shape. After 18 h of co-treatment with LPS, cells exhibited severe modifications of size, shape and density (data not shown) for the highest concentration of MGDGs tested (500μg/mL).

According to these observations, we may consider that, at the cytotoxic concentration of 500 μg/mL, the tested MGDGs promoted the death of the majority of the cells. This observation is not supported by the results of the LDH assay (which indicate cell viability *per se*), but was previously observed and confirmed by other authors using the same cell line, treated in a similar manner (354): while assessing the anti-inflammatory potential of plumbagin on LPS-stimulated Raw 264.7 macrophage cells Pinho and colleagues verified that the LDH could be released from macrophages in an early stage of cells treatment and then degraded during the incubation period, reaching values close to control (354). Moreover, the culture medium DMEM retained its color very close to the initial one, which did not happen in the other wells. DMEM has a pH indicator that changes from red to yellow by acidification, the pH decrease being characteristic of cell metabolism. The fact that no change of the color of the culture medium was observed is another indicative of cell death or metabolic disability.

5.3.3.2. NO released by RAW 264.7 macrophages

The isolated compounds were screened for their ability to affect NO released by LPS-stimulated RAW 264.7 macrophages. All of them showed a dose-dependent NO inhibitory activity. The monoacylglycerol (compound **1**, **Figure 45**) demonstrated lower capacity to inhibit NO production by macrophages than the 1:1 mixture of the MGDGs (compounds **2** and **3**, **Figure 45**) (IC_{50}=65.70 μg/mL *vs* 60.06 μg/mL). The anti-inflammatory reference drug dexamethasone was used as positive control, as inhibitor of NO production by macrophages (**Figure 51**). Macrophage cells were treated with dexamethasone along with the isolated compounds. Like the isolated compounds, the reference anti-inflammatory drug was able to inhibit NO production in a dose-dependent manner (IC_{50} = 34.60 μg/mL) (**Figure 51**).

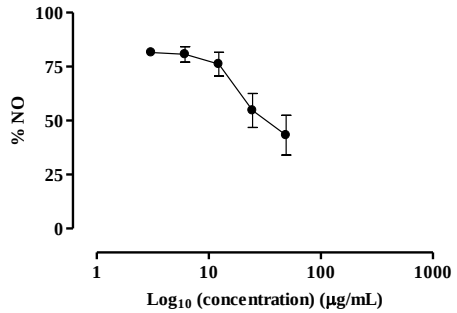

Figure 51. Dexamethasone effect on NO release. Quantification of NO produced by RAW 264.7 cells exposed to dexamethasone and stimulated with 1 μg/mL of LPS for 18 h. Results are expressed in percentage of control with LPS (mean ±SEM of four independent assays performed in duplicate).

According to previous studies (220, 221), galactolipids are able to down-regulate the iNOS protein levels in LPS-stimulated RAW 264.7 cells, suggesting that the decrease in NO production observed with these compounds is related to the down regulation of iNOS expression (220, 221). In these studies it was evaluated the capacity of two MGDGs composed of hexadecatetraenoic acid (C16:4 Ω3) combined with octadecatetraenoic (C18:4 Ω3) and linolenic (C18:3 Ω3) acids to reduce NO production in RAW 264.7 cells and concluded that the compound with more double bonds presented a lower IC_{50}, proving that the levels of unsaturation are related to the anti-inflammatory activity. The same research group reported the NO inhibitory capacity of two MGDGs composed by one glycerol unit linked to a fatty acid and one molecule of galactose. With this study they concluded that the position of the double bonds was more crucial for the bioactivity than the number of double bonds, since compounds with Ω6 fatty acids were more active than Ω3 fatty acids (even having more double bonds) (220, 221).

These findings are in accordance with our results, as both the monoacylglycerol and the MGDGs showed the capacity to reduce NO release in a dose-dependent manner. According to the previous findings (221), the difference between the IC_{50} values of MGDGs fraction and of the monoacylglycerol can be justified by the position of the double bond in the monoacylglycerol at Ω9.

Although the IC_{50} values of our compounds are higher than that of the reference drug, the potential of the monoacylglycerol and the MGDGs as anti-inflammatory agents cannot be set aside. Glucocorticoids are commonly prescribed for the treatment of several inflammatory disorders; nevertheless, there are plenty of undesirable side effects associated with both acute and long term treatments (355). For this reason, glycerolipids and MGDGs may constitute an alternative to common anti-inflammatory drugs, as they present a good therapeutic activity without known side effects. MGDGs have shown a potent anti-inflammatory activity in *in vitro* cultured articular chondrocytes and also the ability to prevent cell proliferation, without affecting cell viability, in osteoarthritic cartilage degeneration (351, 352). Maeda and colleagues went further and investigated the action of MGDGs on colon tumor in mice after an oral administration, demonstrating that these compounds were safe and had a potent antitumor effect (356). These compounds also prove to be able to inhibit croton-oil-induced ear edema in the mouse, with a better IC_{50} than the reference drug (151).

A combination of our study with previous investigations involving similar naturally occurring glyceroglycolipids (151, 221, 352) highlights their anti-inflammatory capacity over different types of cell lines. This contributes to promote their use as potential naturally occurring templates for future drug development.

PART IV

CONCLUSIONS

6. CONCLUSIONS

The work carried out under the subject of this dissertation allowed reaching to the following conclusions:

Seaweeds species under study presented a widely variable sterols profile. The ones of Chlorophyta and Phaeophyta phyla are clearly marked by the abundance of C_{28} and C_{29} sterols, while the ones belonging to Rhodophyta are mainly characterized by their abundance of C_{27} sterols, cholesterol being predominant.

From a health and nutritional point of view, and considering the health effects of C_{28} and C_{29} sterols, Chlorophyta and Phaeophyta are preferred over Rhodophyta. In what concerns to total C_{28} and C_{29} sterols amount, species belonging to Fucales seem to be greatly promising for both introduction in human diets and development of functional foods and additives with cholesterol lowering properties and capacity to reduce and prevent CHD.

Phaeophyta species, particularly *F. spiralis* and the three *Cystoseira* species, revealed substantial concentrations of bioactive phlorotannins.

The purified phlorotannins extracts demonstrated anti-inflammatory activity on RAW 264.7 cells by the reduction of NO, which seems to happen by two different mechanisms: scavenging and inhibition of iNOS.

Purified phlorotannins extracts demonstrated antimicrobial activity over a wide range of pathogenic bacteria and fungi. The extracts were more active against $Gram^+$ bacteria and, among fungi, more active against dermatophytes. The extracts also revealed bactericidal and fungicidal (for dermatophytes) activity, being only fungistatic for yeasts.

Phlorotannins revealed a marked antioxidant activity against NO, $O_2^{\bullet -}$, being also capable of inhibiting LPO and the degradation of HA by the inhibition of HAase.

The isolation of compounds from *F. spiralis* allowed obtaining two MGDGs and a monoacylglycerol, which demonstrated anti-inflammatory properties by the inhibition of NO production by RAW 264.7 cells.

6.1. Practical applications for further research

According to the exposed, phlorotannins demonstrate a huge potential to be used in cosmeceutical preparations with both anti-inflammatory, anti-aging, anti-bacterial and antifungal properties, constituting an alternative to the existing pharmaceutical formulas with undesirable side effects.

Bacteria and inflammation - Acnes vulgaris

Thus, and being aware of the need for further studies, a combination of *F. spiralis* and *C. tamariscifolia* extracts could be interesting for an anti-acne formulation, since it would be possible to combine the anti-bacterial effect of *F. spiralis* with the anti-inflammatory effect of *C. tamariscifolia*, taking into account the inflammatory conditions associated with acne.

Fungi – superficial dermatophytosis and disseminated infections

On the same direction, a *C. nodicaulis* based formulation would be interesting for the treatment of dermatophytosis, considering its fungicidal effect. On the other hand, *F. spiralis* could be an alternative for the treatment of disseminated candidiasis, considering its ability to reduce *C. albicans* virulence factor.

Enzymes and oxidants - Aging

Considering its capacity to radicals scavenging, for LPO inhibition and HA break prevention by the inhibition of HAase, all common reactions in the aging process, *F. spiralis* seems to be a powerful matrix for the development of an anti-aging formula. Nevertheless, and considering that aged skins are more sensitive and reactive, frequently presenting allergic reactions, this matrix could have a dual effect and prevent the allergic reactions *via* HAase inhibition.

Glycerolipids - Anti-inflammatory drugs development

The MGDGs and the monoacylglycerol isolated from *F. spiralis* demonstrated anti-inflammatory capacity. Despite not being better than the reference drug dexamethasone, it is still encouraging, considering the undesirable side effects of corticoids. Additionally, the isolated molecules can constitute a starting point for molecular design, in order to reach new molecules with a powerful anti-inflammatory activity and irrelevant side effects.

PART V

REFERENCES

7. REFERENCES

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